



**Universidade Nova de Lisboa
Instituto de Higiene e Medicina Tropical**

**Improved nucleic acid testing strategies to
detect and discriminate veterinary relevant
Mycobacterium tuberculosis complex
members**

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**Improved nucleic acid testing strategies to detect and
discriminate veterinary relevant *Mycobacterium*
tuberculosis complex members**

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To my parents.

“Nowhere in these ancient communities of the Eurasian land mass, where it is so common and feared, is there a record of its beginning. Throughout history, it had always been there, a familiar evil, yet forever changing, formless, unknowable. Where other epidemics might last weeks or months, where even the bubonic plague would be marked forever afterwards by the year it reigned, the epidemics of tuberculosis would last whole centuries and even multiples of centuries. Tuberculosis rose slowly, silently, seeping into homes of millions, like an ageless miasma. And once arrived, it never went away again. Year after year, century after century, it tightened its relentless hold, worsening whenever war or famine reduced the peoples' resistance, infecting virtually everybody, inexplicably sparing some while destroying others, bringing the young down onto their sickbeds, where the flesh slowly fell from their bones and they were consumed in the yearslong fever, their minds brilliantly alert until, in apocalyptic numbers, they died, like the fallen leaves of a dreadful and premature autumn.”

The Forgotten Plague:
How the War against Tuberculosis was Won - and Lost
Frank Ryan, 1992

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Resumo

Improved nucleic acid testing strategies to detect and discriminate veterinary relevant *Mycobacterium tuberculosis* complex members

Palavras-chave: complexo *Mycobacterium tuberculosis*; *Mycobacterium bovis*; tuberculose bovina; testes de DNA; diagnóstico molecular.

Os membros do complexo *Mycobacterium tuberculosis* (MTC) são agentes causadores de tuberculose em humanos e animais. A tuberculose bovina tem sido sujeita nas últimas décadas a programas de erradicação bastante dispendiosos, na maioria dos países desenvolvidos, envolvendo a análise laboratorial de tecidos de animais suspeitos para a detecção dos membros do MTC, nomeadamente *Mycobacterium bovis*. O diagnóstico definitivo é obtido através da cultura bacteriológica, o que pode levar 6-12 semanas, período durante o qual a carcaça do animal suspeito e a exploração de origem permanecem sob embargo sanitário. Neste trabalho, descreve-se um protocolo de extracção de DNA de fácil utilização adaptado aos tecidos, o qual é acoplado a um *semi-nested* PCR em tempo real, utilizando como alvo a IS6110, por forma a melhorar a detecção directa de bactérias pertencentes ao MTC em animais, abreviando o período necessário ao diagnóstico. O ensaio foi avaliado num grupo de 128 amostras de tecido provenientes de bovinos, javalis, veados e raposas. O desempenho global do teste corresponde a uma sensibilidade e especificidade de diagnóstico de 98,2% e 88,7%, respectivamente. Foi observado um coeficiente *kappa* de 0,859 entre o ensaio de *semi-nested* PCR e a cultura bacteriológica. Este ensaio permite a detecção rápida de micobactérias tuberculosas em amostras de animais com alta sensibilidade e especificidade, sendo acessível e de baixo custo para uma utilização num laboratório de diagnóstico veterinário.

As espécies do MTC são geneticamente muito semelhantes, mas podem divergir na sua epidemiologia, nomeadamente na distribuição geográfica e preferência pelo hospedeiro, factores de virulência e padrões de susceptibilidade antimicrobiana. No entanto, o diagnóstico laboratorial convencional não diferencia rotineiramente as espécies do MTC. Foi desenvolvido um algoritmo de identificação rápido e robusto, baseado em PCR em tempo real, dirigido para cinco alvos genómicos para a identificação das espécies do MTC vulgarmente associadas à tuberculose nos bovinos e outros animais. O primeiro passo permite a confirmação dos membros do MTC nas culturas, através da detecção da IS6110, ou como uma espécie micobacteriana, pela presença do 16S rDNA. Se uma espécie do MTC for identificada, o segundo passo do algoritmo permite avaliar a presença ou ausência das regiões genómicas RD1, RD4 e RD9. O padrão correspondente permite inferir a espécie do isolado como *M. tuberculosis* (se todas as RDs estiverem presentes), *M. caprae* (se apenas a RD1 e RD4 estiverem presentes) ou *M. bovis* (se apenas a RD1 estiver presente). O algoritmo de identificação desenvolvido demonstrou um coeficiente *kappa* de 0,970 com o resultado da análise bacteriológica. O ensaio pode ser implementado em laboratórios de diagnóstico veterinário, especialmente em laboratórios de referência.

Tem-se registado uma procura crescente por métodos de diagnóstico de doenças infecciosas rápidos, de fácil utilização e acessíveis, passíveis de serem utilizados em pontos-de-decisão. A detecção dos membros do MTC é geralmente realizada por diversos métodos convencionais baseados na cultura, que normalmente necessitam de oito semanas. Foram também desenvolvidas estratégias de diagnóstico molecular, mas a maioria requer operadores qualificados e equipamentos e infra-estruturas sofisticadas. Recentemente, a técnica de *Loop-Mediated Isothermal Amplification* (LAMP) mostrou-se promissora para o desenvolvimento de testes rápidos, de baixo custo, sensíveis e específicos para a detecção de agentes patogénicos. Neste trabalho, foram optimizados dois sistemas LAMP em formato *duplex* (dLAMP) para a identificação do MTC e *Mycobacterium tuberculosis*, e do MTC e *M. bovis*, apresentando valores de sensibilidade e especificidade comparáveis a outras abordagens em que se utiliza o PCR convencional. Os resultados das amplificações são avaliados colorimetricamente utilizando dispositivos de fluxo lateral, simples e comercialmente disponíveis, para a detecção de ácidos nucleicos (NALF).

Os resultados apresentados nesta dissertação contribuem para a melhoria das estratégias de diagnóstico molecular existentes no combate à tuberculose animal.

Abstract

Improved nucleic acid testing strategies to detect and discriminate veterinary relevant *Mycobacterium tuberculosis* complex members

Keywords: *Mycobacterium tuberculosis* complex; *Mycobacterium bovis*; bovine tuberculosis; nucleic acid testing; molecular diagnosis

Members of *Mycobacterium tuberculosis* complex (MTC) are causative agents of tuberculosis in both humans and animals. Bovine tuberculosis has been tackled for decades by costly eradication programs in most developed countries, involving the laboratorial testing of tissue samples from allegedly infected animals for detection of MTC members, namely *Mycobacterium bovis*. Definitive diagnosis is usually achieved by bacteriological culture, which may take up to 6-12 weeks, during which the suspect animal carcass and herd are under sanitary arrest. In this work we describe a user-friendly DNA extraction protocol adapted for tissues and coupled with an IS6110-targeted semi-nested real-time PCR assay to enhance the direct detection of MTC bacteria in animal specimens, reducing the time to achieve a diagnosis. The assay was evaluated with a group of 128 fresh tissue specimens collected from bovines, wild boars, deer and foxes. Overall, the full test performance corresponds to a diagnostic sensitivity and specificity of 98.2% and 88.7%, respectively. An observed kappa coefficient was estimated in 0.859 for the overall agreement between the semi-nested PCR assay and the bacteriological culture. This novel IS6110-targeted assay allows the fast detection of tuberculous mycobacteria in animal specimens with very high sensitivity and specificity, being amenable and cost effective for use in the routine veterinary diagnostic laboratory with further automation possibilities.

MTC species are genetically very similar but may differ in their epidemiology, namely geographic distribution and host preferences, virulence traits and antimicrobial susceptibility patterns. However, the conventional laboratory diagnosis does not routinely differentiate between the species of the MTC. We developed a rapid and robust two-step five-target probe-based real-time PCR identification algorithm, based on genomic deletion analysis, to identify the MTC species most commonly associated with TB in livestock and other animals. The first step allows the confirmation of the cultures as MTC members, by targeting their IS6110 element, or as a mycobacterial species, if only a 16S rDNA product is detected in the duplex amplification reaction. If a MTC member is identified, the second amplification step allows to assess the presence or absence of the RD1, RD4 and RD9 genomic regions. The correspondent pattern allows to infer the species of the isolate as *M. tuberculosis* (if all RDs are present), *M. caprae* (if only RD1 and RD4 are present) and *M. bovis* (if only RD1 is present). The identification algorithm developed presented an almost perfect agreement with the results of the routine bacteriological analysis, with a kappa coefficient of 0.970. The assay is able to be adaptable to automation and implementation in the routine diagnostics framework of veterinary diagnostics laboratories, with a particular focus for reference laboratories.

Rapid, user-friendly and affordable diagnostic tests for use in the point-of-decision or point-of-care settings are in high demand globally. Detection of MTC members is generally performed by cumbersome conventional culture-based methods, which usually takes up to eight weeks. Molecular diagnosis strategies were also developed but most of these requires skilled operators and sophisticated equipments and facilities. More recently, the Loop-Mediated Isothermal Amplification (LAMP) technique showed promise for the development of rapid, low-cost, sensitive and specific tests for detecting pathogens. In this work we have optimized duplex LAMP (dLAMP) assays for the identification of MTC and *Mycobacterium tuberculosis*, and MTC and *M. bovis*, presenting similar sensitivities and specificities when compared to standard PCR approaches. The amplification results are assessed colorimetrically by using simple and commercially available nucleic acid lateral flow (NALF) strips.

With the work described in this dissertation we aim to modestly contribute to the improvement of the existing molecular diagnosis strategies to combat animal tuberculosis.

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List of abbreviations

Af1 - African 1 Complex

Af2 - African 2 Complex

AFB - Acid Fast Bacilli

Ag - Silver

Au - Gold

AuNPs - Gold Nanoparticles

BAAR - Acid-Alcohol Resistant Bacilli

BCG - Bacillus Calmette-Guérin

Bst - *Bacillus stearothermophilus*

bTB - Bovine Tuberculosis

CPC - Hexadecyl Pyridine Chloride

C_t – Threshold cycle

DGAV - *Direcção Geral de Alimentação e Veterinária*

DGS - *Direcção Geral de Saúde*

DIG – Digoxigenin

DNA - Deoxyribonucleic Acid

DR - Direct Repeat

dsDNA - Double Stranded DNA

DVR - Direct Variable Repeat

EDTA - Ethylenediamine tetraacetic acid

ELISA - Enzyme-Linked Immunosorbent Assay

ESAT-6 - 6 kDa early secretory antigenic target

Eu1 - European 1 Complex

Eu2 - European 2 Complex

FITC - Fluorescein Isothiocyanate

FRET - Fluorescence Resonance Energy Transfer

GC - Guanine and Cytosine

gyrB - Coding gene for the B subunit of DNA gyrase

HIV - Human Immunodeficiency Virus

HPLC - High-Performance Liquid Chromatography

List of abbreviations (Cont.)

IFN- γ - Interferon-Gamma

IHMT/UNL - *Instituto de Higiene e Medicina Tropical/Universidade Nova de Lisboa*

INIAV - *Instituto Nacional de Investigação Agrária e Veterinária*

IS - Insertion Sequence

IVD - *In Vitro* Diagnostics

LAMP - Loop-Mediated Isothermal Amplification

LFD – Lateral Flow Dipstick

LNIV - *Laboratório Nacional de Investigação Veterinária*

LSPs - Large Sequence Polymorphisms

M. - *Mycobacterium*

MIRU - Mycobacterial Interspersed Repetitive Units

MTC - *Mycobacterium tuberculosis* complex

NAT - Nucleic Acid Testing

NPs - Nanoparticles

NPV - Negative Predictive Value

OIE - World Organisation for Animal Health

OMS – *Organização Mundial de Saúde*

PBS - Phosphate Buffered Saline

PCR - Polymerase Chain Reaction

POC - Point-of-Care

PPD - Purified Protein Derivative

PPV - Positive Predictive Value

Pt - Platinum

RD - Region of Difference

RFLP - Restriction Fragment Length Polymorphism

RNA – Ribonucleic Acid

rRNA - Ribosomal Ribonucleic Acid

SDS - Sodium Dodecyl Sulfate

SICCT - Single Intradermal Cervical Tuberculin Test

SNPs - Single Nucleotide Polymorphisms

List of abbreviations (Cont.)

Spoligotyping - Spacer Oligonucleotide Typing

SPR - Surface Plasmon Resonance

Taq - *Thermus aquaticus*

TB - Tuberculosis

TCH - Thiophene-2-carboxylic acid hydrazide

VLA - Veterinary Laboratories Agency

VNTR - Variable Number Tandem Repeats

WHO - World Health Organization

List of Units

bp – base pair

°C – degrees Celsius

rpm – rotation per minute

msec – millisecond; sec – second; min – minute

nm – nanometer

μl – microliter; ml – milliliter

fg – femtogram; pg – picogram; ng – nanogram; μg – microgram

pmol – picomole

μM – micromolar

nM – nanomolar; mM – millimolar; M – molar

CHAPTER 1

General Introduction

1.1. Tuberculous mycobacteria and tuberculosis

Tuberculosis (TB) is an epidemic and serious infectious disease of global proportions, responsible for the death of approximately two million people per year, being estimated that one third of the world's population is latently infected [1]. Although curable, tuberculosis is still the leading cause of death in humans in many countries, and increasing numbers of cases of disease are reported in many regions of the world. The disease also affects animals, particularly livestock, with tremendous economic impacts. This disease is thus still a serious public health problem [1]. Despite being one of the most documented infectious diseases since the first records of the mankind [228], the main etiological agent of human tuberculosis, the Koch bacillus, was discovered by Robert Koch only in 1882 [2]. Today, it is known that tuberculosis is caused by several members of the *Mycobacterium tuberculosis* complex (MTC), a group of phylogenetically-related species including: *M. tuberculosis*, the principal agent of human tuberculosis; and *M. bovis* and *M. caprae*, most associated with tuberculosis in cattle and goats, respectively [3]. In the genus *Mycobacterium*, besides the MTC members, there are more than 120 recognized species, of which at least 20 to 30 may cause disease in humans [4] (Table 1.1).

Table 1.1. Mycobacteria species frequently found in Medicine, according to the risk of infection (adapted from [6]).

Classification	Species
Strict pathogens	<i>M. tuberculosis</i> ; <i>M. bovis</i> ; <i>M. africanum</i> ; <i>M. leprae</i> ; <i>M. ulcerans</i> ; <i>M. szulgai</i> ; <i>M. marinum</i>
Potential or opportunistic pathogens	<i>M. avium</i> ; <i>M. intracellulare</i> ; <i>M. scrofulaceum</i> ; <i>M. kansasii</i> ; <i>M. xenopi</i> ; <i>M. haemophilum</i> ; <i>M. genavense</i> ; <i>M. simiae</i> ; <i>M. malmoeense</i> .
Rare pathogens, commensal or saprophytic	<i>M. fortuitum</i> ; <i>M. peregrinum</i> ; <i>M. chelonae</i> ; <i>M. abscessus</i> ; <i>M. thermoresistibile</i> ; <i>M. gordonae</i> ; <i>M. triviale</i> ; <i>M. gastri</i> ; <i>M. terrae</i> ; <i>M. flavescens</i> (among others)

1.1.1. Classification and general characteristics of mycobacteria

The genus *Mycobacterium* is one of the oldest bacterial genus described. The formal classification of mycobacteria started in 1896 when Lehmann and Neumann proposed for the first time the creation of the genus *Mycobacterium*, encompassing a number of microorganisms whose growth, forming branching filamentous forms on the surface of the liquid culture media, was similar to fungi (the Greek prefix "myco" means "fungus") (Figure 1.1).

Mycobacteria are dispersed in nature, either as saprophytic or as pathogenic species, having the last ones a wide range of hosts, ranging from plants to humans (Table 1.1). The genus *Mycobacterium* belongs to the family *Mycobacteriaceae* of the order *Actinomycetales* and, as mentioned above, includes more than 120 validly described species [4, 5]. Some of these species are strict or opportunistic (or potential) pathogens that affect both humans and animals. Among the strict pathogens, the principal species affecting humans include *Mycobacterium tuberculosis*, the causative agent of tuberculosis, *M. leprae*, which causes leprosy, and *M. bovis* that causes tuberculosis in animals but can also cause disease in humans. Opportunistic pathogens comprise a variety of mycobacterial species, including *M. avium* and *M. kansasii*, among others [6]. *Mycobacteria* are Gram-positive, rod-shaped, aerobic, non-motile, non-sporulating, and requiring aerobic conditions for their growth. Its optimum temperature for growth varies between 30 °C and 45 °C [6]. One peculiar characteristic of the genus consists in the chemical and structural complexity of the cell wall that is unique among prokaryotes, comprising a high percentage of long-chain lipids, including mycolic acids of high molecular weight with 60 to 90 carbon atoms [7].



Figure 1.1. Colonies of *Mycobacterium tuberculosis*, strain H37Ra, grown on Middlebrook 7H10 solid medium (original from Pedro Costa).

Among other features, the mycobacterial cell wall gives the property of acid-alcohol resistance and mainly contributes to the hydrophobic nature of the cells surface, which leads to the formation of lumps when grown in liquid medium. The differences in the number of carbon atoms, and of their chemical bonds, in mycolic acids are important for the classification of mycobacteria. Other microorganisms such as *Corynebacterium*, *Nocardia* and *Rhodococcus* also produce mycolic acids, although with different molecular structures and lower molecular weight [6].

The different growth rates in specific culture media have led to the traditional division of mycobacteria into two groups: the rapidly growing and the slow growing mycobacteria (Figure 1.2). This traditional division scheme distinguishes mycobacteria that grows in less than seven days, without additional growth factors, from those whose growth occur after seven days and requiring additional growth factors added to the media. The first group includes for example saprophytic species such as *M. smegmatis* and *M. fortuitum*. The second slow-growing group encompasses pathogenic species, including *M. tuberculosis*, *M. bovis*, *M. avium* subsp. *avium* and *M. avium* subsp. *paratuberculosis*. The identification of mycobacteria is conventionally performed on the basis of their phenotypic characteristics, including morphological, cultural and biochemical features [6].

The pathogenic mycobacteria were initially separated into two groups, the *Mycobacterium tuberculosis* complex (MTC) and the non-MTC, or atypical,

mycobacteria species, being their discrimination performed according to phenotypic identification criteria in the majority of clinical diagnostic laboratories [129].

The evolution of genomic studies have permitted to establish relationships between the genetic and phenotypic characteristics of mycobacteria, e.g., leading to the genetic differentiation between the slow and fast growing species and to the description of new species and reclassification of the existing ones [8] (Figure 1.2). Overall, the members of the genus *Mycobacterium* have a high content of guanine and cytosine in genomic DNA, in the order of 62% to 70% (except for *M. leprae*, which is about 58%), and share, at least, 94.3% of similarity in the sequences of the 16S ribosomal gene [6].

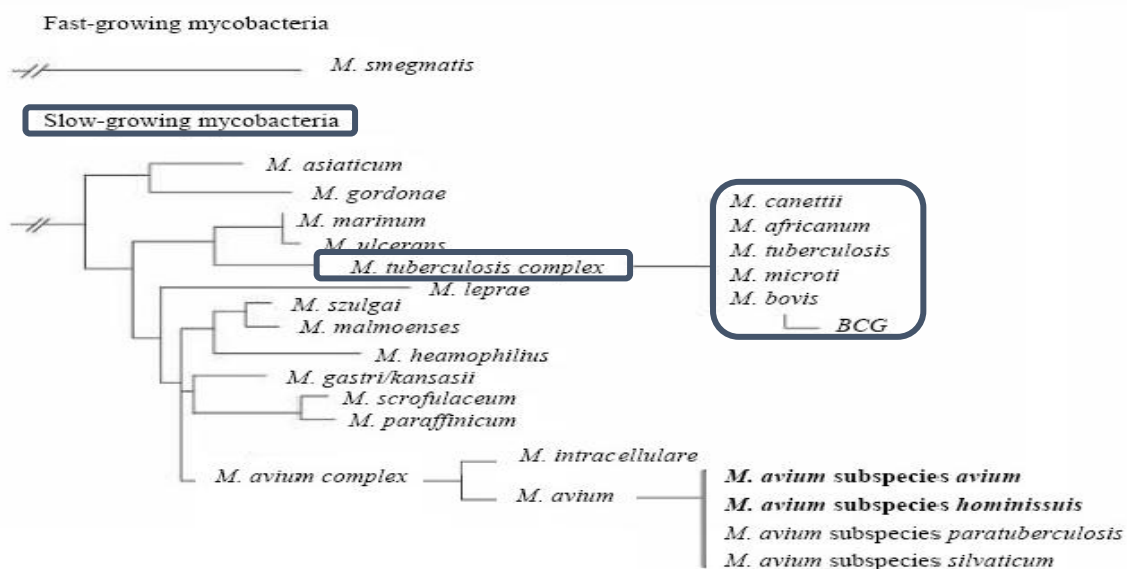


Figure 1.2. Phylogenetic tree of mycobacteria based on the 16S rRNA gene sequences (Adapted from [9]).

1.1.2. The *Mycobacterium tuberculosis* complex

The species of *Mycobacterium* that are agents of tuberculosis (tuberculous mycobacteria) are gathered in the so-called *Mycobacterium tuberculosis* complex (MTC). These species share about 99.95% of sequence homology, with a greatly reduced genetic diversity at the nucleotide level, and present identical sequences in the 16S ribosomal gene, [10, 11]. The genome of the MTC members is longer than in other intracellular microorganism's genomes, with approximately 4.4 million base pairs, which reflects the biochemistry complexity of its life cycle as facultative intracellular

parasites, and also contains a higher GC content of about 65%. The exchange of genetic material, such as horizontal gene transference, is virtually non-existent in these species which results mainly in a clonal evolution, relying on extensive deletions of the nucleotide sequences (LSPs, Large Sequence Polymorphisms), rearrangements and chromosome point mutations [3, 12].

The MTC is currently constituted by various species and subspecies with human and veterinary clinical importance: *M. canettii*, *M. africanum*, *M. pinnipedii*, *M. microti*, *M. caprae*, *M. bovis*, *M. tuberculosis*, *M. mungi*, *M. orygis* and the Dassie bacillus [13-17]. MTC mycobacteria are strict pathogens, depending on the host for its survival. Despite of the high degree of conservation of their genomes, the MTC species demonstrate important phenotypic differences, adaptations to different hosts and show different degrees of virulence [18]. For example, *M. tuberculosis*, the main agent of human tuberculosis, is almost always associated with the human. Otherwise, *M. bovis* is found primarily associated with cattle, *M. caprae* to goats and *M. pinnipedii* to marine mammals [18]. However, the adaptation of these species to each host is not necessarily strictly as it is for other pathogenic bacteria. All members of the MTC can cause disease in humans and other mammals that were not initially considered as primary hosts (Table 1.2) [4]. It is noteworthy that *M. bovis* and *M. caprae* represents a significant potential of zoonotic transmission to Human [19, 20]. There is evidence pointing to the possibility of person-to-person transmission of the zoonotic *Mycobacterium* species [21], but the main routes of transmission are the contact with infected animals and intake of dairy improperly pasteurized or unpasteurized [1, 22]. *Mycobacterium bovis* species is responsible for between 0.5 and 7.2% of human tuberculosis cases in industrialized countries and it is estimated that it is responsible for 10 to 15% of new cases in developing countries [23]. Studies on the prevalence of human tuberculosis caused by these species are scarce in Portugal.

Table 1.2. Species belonging to the *Mycobacterium tuberculosis* complex and respective hosts.

Species	Primary Host	Other Hosts	Characteristics
<i>M. tuberculosis</i>	Human	Other mammals	Species almost exclusively found in humans. However, there are records of infections in other mammals [24-28].
<i>M. bovis</i>	Cattle	Sheep, goats, dogs, cats and humans	Species with the highest number of possible hosts [6, 29-32].
<i>M. caprae</i>	Goats	Human	Formerly considered a subspecies of <i>M. bovis</i> , but genetic differences have led to it being considered a distinct species [33].
<i>M. africanum</i>	Human	-	Species phenotypically intermediate between <i>M. tuberculosis</i> and <i>M. bovis</i> and whose area of focus is almost exclusively restricted to the African continent. Two varieties were described [34].
<i>M. microti</i>	Rodents	Human	Pathogenicity level lower than the remaining species, being a potential substitute for BCG vaccine strains [35-37].
<i>M. canettii</i>	Human	-	Species most divergent from MTC and allegedly nearest the ancestral species precursor of the complex [3, 38, 39].
<i>M. pinnipedii</i>	Seals	Humans and other mammals	Species first isolated in seals in South America with lesions of tuberculosis [40] and has also been associated with cases of tuberculosis in mammals in other zoos [41].
<i>M. mungi</i>	Banded mongooses	-	The causative TB agent among banded mongooses, first isolated in Botswana. Host spectrum and transmission dynamics remain unknown [13].
Dassie bacillus	Hyrax or Dassie	-	An infrequent variant of the <i>M. tuberculosis</i> complex characterized as being most similar to <i>M. microti</i> , is the causative agent of tuberculosis in the dassie (<i>Procavia capensis</i>) [42].
<i>M. orygis</i>	?	Mammals	Oryx bacilli have been isolated from members of the <i>Bovidae</i> family, i.e., oryxes, gazelles, deer, antelope, and waterbucks, although their exact host range remains unsettled. No human disease caused by the oryx bacilli has been reported [14].

Phylogenetic relationships and evolutionary scenario

As mentioned above, the MTC members share 99.95% of genetic homology, with a greatly reduced genetic diversity at the nucleotide level. Although genetically very similar, the members of the complex can be distinguished from each other by stable molecular differences, such as deletions or single nucleotide polymorphisms (SNPs), which have been the basis for evolutionary studies of these mycobacteria [3, 43]. The irreversible deletions of chromosomal regions, called Regions of Difference (RDs), were recognized in a much larger number in the genome of *M. bovis* compared to other members of the complex [3]. The analysis of these deletions allowed Brosch and collaborators [3] to outline a new phylogenetic tree for the MTC, later on confirmed by another independent study [11], in which *M. bovis* and *M. bovis* BCG appear as the last descendants of the complex (Figure 1.3). The vaccine strains of *M. bovis* BCG still exhibit an additional deletion in RD1, exclusive of these strains, and whose absence is associated with lower virulence [44]. The various members of the MTC that infect animals have in common the absence of RD9, which is present in the other members, like *M. tuberculosis* [3]. Other genomic data, including that resulting from the whole-genome sequencing of *M. bovis* [45] and *M. tuberculosis* [46], corroborate the evolutionary scenario proposed by Brosch *et al.* (2002) for the MTC, in which *M. bovis* was the last descendant in the phylogeny (Figure 1.3). This new hypothesis came to oppose the previous one, based on epidemiological evidence, that *M. bovis* was the precursor species due to its wider hosts preferences range, and have served as a basis for the evolution of *M. tuberculosis*, by adaptation phenomena and specialization to Human host [47].

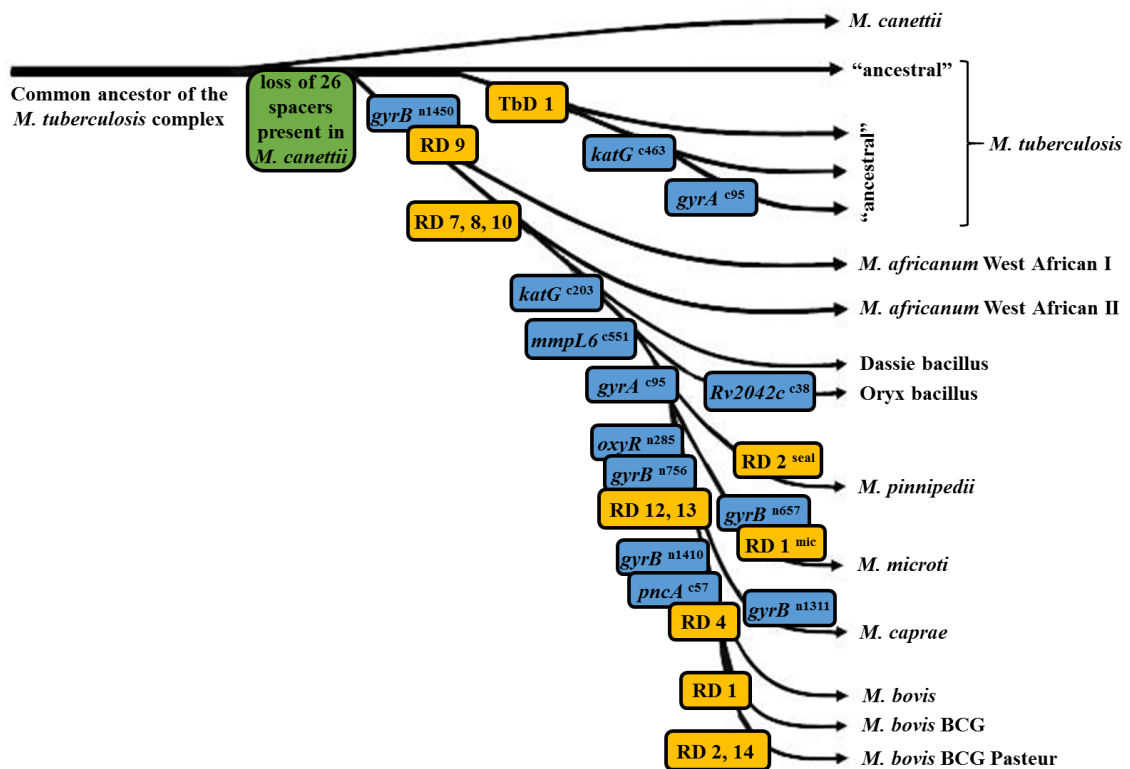


Figure 1.3. Phylogeny of the *Mycobacterium tuberculosis* complex based on the analysis of Regions of Difference and Single Nucleotide Polymorphisms. Each orange box represents a deletion of one or more specific RDs in a given species [4]. Blue boxes indicate the SNPs; superscripts mark the position of the mutation at either the nucleotide (n) or the codon (c) of the respective genes. Adapted from [3], including information from [4, 14, 17, 50, 158].

Other phylogenies were produced for the MTC using additional molecular markers, such as deletions of spacer sequences of the Direct Repeat (DR) region and the identification of Single Nucleotide Polymorphisms (SNPs) [48-52], which are consistent with the proposal presented by Brosch *et al.* (2002). In 2009, in an overall phylogenetic analysis of the MTC bringing together all these molecular markers, this group of microorganisms appears as a set of ecotypes adapted to different hosts, corresponding the different affinities of the host to different niches (Figure 1.4) [4, 53]. The MTC members harbouring animals as their main hosts share the absence of RD9, which is present in *M. tuberculosis*, and also other common features (e.g. deletion of specific DR spacer sequences and SNPs).

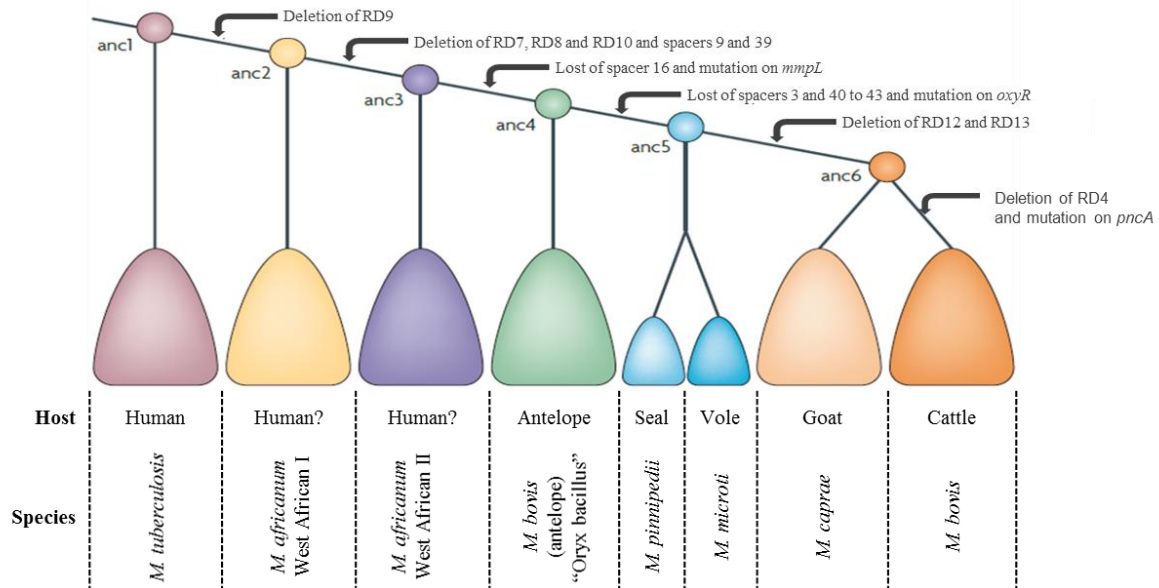


Figure 1.4. Evolution of members of *Mycobacterium tuberculosis* complex lacking the RD9. The predecessor species are numbered from Anc1 to Anc6 (Adapted from [4, 53]).

Clonal complexes of *Mycobacterium bovis*

Bovines are the main hosts of *M. bovis*, which determines the disease called bovine tuberculosis (bTB). This term is also often used to describe the infection by *M. bovis* in other species, including wild animals and humans, to demonstrate the cattle as a source of infection. Although bovine tuberculosis has been reported on every continent where there are breeding of cattle, it was assumed to have originated in a particular geographic location, and since then has spread throughout the globe [54]. Historical data indicate that bovine tuberculosis has its origins in Europe and, especially during the colonial period, was distributed from that continent to the rest of the world. Myers and Steele (1969) suggested that *M. bovis* reached the northern Italy and from there moved to Western Europe and the UK. Webb (1936) also reasoned that the spread through the world was favoured by the export of infected cattle originated from the UK (mainly) and the Netherlands to their respective colonies [55]. The intensification of the dairy industry in combination with the movement of bovine animals has contributed to the

transmission of *M. bovis*, especially in the absence of appropriate control and eradication measures [56].

Modern methods of typing, based on the analysis of genetic characteristics, have determined progress in the knowledge of the geographical distribution of *M. bovis* and allowed the identification of dominant clonal complexes within broader geographic areas [57]. The cattle trade between neighbouring regions and countries leads to the dispersion of *M. bovis* and the dominance of clonal complexes in large areas [58]. These clonal complexes were mainly defined by the presence of specific patterns of deletions, namely in the DR region, by a reverse-hybridization typing technique named spoligotyping. Phylogeographic analysis have confirmed the existence of four major *M. bovis* clonal complexes: African 1 (Af1), African 2 (Af2), European 1 (Eu1) and European 2 (Eu2) [54, 58-60]. Two of these complexes are geographically located in Africa (Af1 and Af2), a third complex exhibit a global distribution (Eu1), and the fourth complex has a strong geographical predominance in the Iberian Peninsula (Eu2). The Af1 clonal complex (dominant in Cameroon, Nigeria, Mali and Chad) and the Af2 complex (common in East Africa) were identified by Berg *et al.* (2011) and Muller *et al.* (2009), the clonal complex Eu1 (common in Ireland, UK and overseas countries) was identified by Smith *et al.* (2011), and the clonal complex Eu2 (predominantly in the Iberian Peninsula) was described by Rodriguez-Campos *et al.* (2012).

Most members of the Af1 clonal complex are characterized by a specific chromosomal deletion (RD Af1) and the absence of the DR spacer 30 by spoligotyping. The RD Af1 deletion is frequently found in isolates from Mali, Cameroon, Nigeria and Chad, although this deletion was not observed in other African countries such as Algeria, Burundi, Ethiopia, Madagascar, Mozambique, South Africa, Tanzania and Uganda [58]. The Af2 clonal complex is characterized by the chromosomal deletion RD Af2 and by the absence of spacers 3-7 in the spoligotyping pattern. This complex was identified with high frequency in isolates from Uganda, Burundi, Tanzania and Ethiopia. The RD Af2 deletion was not detected in isolates from Algeria, Mali, Chad, Cameroon, South Africa and Mozambique, suggesting that the Af2 clonal complex is located in East Africa. The absence of spacers 3-7 was rarely observed in isolates from outside Africa and the few isolates detected showed an intact RD Af2. An interesting fact is that Af2 *M. bovis* members mostly possess four or more copies of the IS6110, in contrast to

most other strains that have only one or fewer copies. The deletion RDaf2 were intact in Af1 clonal complex strains, as well as RDaf1 deletion showed intact for the Af2 strains, allowing to conclude that the strains of clonal complexes Af1 and Af2 are mutually exclusive and do not share any phylogenetic history with the latest common ancestor of each clonal complex, suggesting that the mixing between the cattle populations in that countries is uncommon [54, 59].

The clonal complex Eu1 is characterized by the deletion of the chromosomal region RDEu1 and was identified by the analysis of spoligotyping patterns available in the database of *M. bovis* (www.mbovis.org). Over 1000 strains from over 30 countries were analysed. This complex is frequently found in the Republic of Ireland and UK, represent less than 14% of the isolates in France, Spain and Portugal and proved rare in other European Community countries and Iran. However, Eu1 complex was found with high frequency in countries that had cattle trade relations with the UK (USA, South Africa, New Zealand, Australia and Canada). In the American continent, with the exception of Brazil, the Eu1 was also found with high frequency in Argentina, Chile, Ecuador and Mexico. Isolates from Korea and Kazakhstan were also associated with Eu1 clonal complex. The simplest explanation for the worldwide distribution of Eu1 is that it was spread by infected cattle from the UK, such as the Hereford cattle breed created in the 18th century, to old trading partners, although there is no evidence of secondary spread since then. This was the first identification of a clonal complex of *M. bovis* globally dispersed, which tries to explain much of the current global distribution of this major causative agent of bTB [54]. The Eu2 clonal complex, unlike the other clonal complexes mentioned above, is characterized by the absence of the spacer 21 and by a SNP in the *guaA* gene [61]. The Eu2 isolates of *M. bovis* are from the Iberian Peninsula, where they are found with high frequency. Eu2 is also found in France, Italy and the British Isles but with a very low frequency. Previous studies have revealed that about 70% of the strains of *M. bovis* in Portugal [62] and Spain [63] were characterized by the absence of spacer 21.

In summary, the MTC includes a highly clonal group of strains [53] and the genetic diversity found among its members is in part caused by genomic deletions that should represent diverse genetic events [64]. These deletions, termed large sequence polymorphisms (LSPs), have been explored in an attempt to disclose a new evolutionary

scenario for the MTC, based on regions of difference [3]. SNPs have also been used, however with a more targeted application for phylogenetic studies [39, 53]. The analysis of the clonal complexes of *M. bovis* currently represents a new tool for molecular epidemiologists, allowing the creation of hypotheses to investigate the demographic distribution and the pathogenicity of these important zoonotic agents of global significance [53].

1.2. Bovine tuberculosis

As mentioned above, *M. bovis* is the major etiological agent of bovine tuberculosis, a most important chronic and debilitating animal disease. Besides cattle, *M. bovis* can also cause tuberculosis in other mammalian species, including humans. Like human tuberculosis, bovine tuberculosis has a worldwide distribution and importance. It is a zoonosis with a high socio-economic impact, due to the low productivity of cattle and imposed restrictions on animal trade and products thereof, as well as costs associated with the implementation of programs to control and eradicate the disease, consisting also a major risk to public health [65, 66]. The disease has important implications in public health, especially in developing countries [1, 67].

Bovine tuberculosis has a predominantly chronic clinical evolution, with the development of typical granulomatous lesions, mainly in the lungs and lymph nodes, although any organ can be affected. The main routes of infection of bovine tuberculosis in animals are the direct infection by inhalation, ingestion and, less frequently, by congenital route. The mode of transmission more common is by aerosols inhalation, also due to the high survival rate of the agent in aerosols [68]. The infection by indirect route can also occur since *M. bovis* is resistant to adverse environmental conditions, surviving for more than 74 days in fomites and from two to six months in faeces, depending on the temperature and humidity conditions [69]. The anatomical location of the lesions suggests the most likely route of infection: hepatic lesions indicate congenital infection, intestinal lesions the infection by the gastrointestinal tract and lesions in the respiratory route the infection by aerogenous dissemination.

The disease, despite having been eradicated or effectively controlled in most developed countries, continue to cause major problems in livestock in a large number of

developing countries [1, 22, 70]. In Portugal, every year, the costs are high, resulting from the compensation to producers, slaughter of infected animals and initiatives to control and eradicate the disease. Despite the efforts made by the veterinary authorities, the eradication of bovine tuberculosis has not yet been achieved in Portugal, although the prevalence is low in the European context. A decrease of the incidence of bovine tuberculosis has been recorded since 1997 [71, 72]. In 2011, the reported prevalence in cattle was 0.17% against 0.07% and 0.09% in 2001 and 2002, respectively. As we can see in Table 1.3, there has been an apparent upsurge in the prevalence of the disease in Portugal, also potentially related with the maintenance of *M. bovis* in wildlife [104, 229].

Table 1.3. Prevalence of bovine tuberculosis in Portugal (Adapted from [72]).

Year	Total number of animals tested	Number of positive animals	% Positive animals (Animal prevalence)
2001	783.680	546	0.07
2002	776.231	716	0.09
2003	958.306	1.221	0.13
2004	984.527	856	0.09
2005	976.532	647	0.07
2006	976.893	425	0.04
2007	1.006.908	414	0.04
2008	1.032.586	264	0.03
2009	1.060.831	885	0.08
2010	1.036.310	2.702	0.26
2011	1.063.167	1.835	0.17

1.2.1. Implications for public health

Since ancient times that *M. bovis* infects man [73], although *M. tuberculosis* is the main causative agent of human tuberculosis. In the early twentieth century, cases of tuberculosis caused by *M. bovis* in humans showed up as extrapulmonary forms, from consumption of unpasteurized milk and affecting mainly children [74]. The prevalence of human tuberculosis caused by *M. bovis* decreased dramatically in countries where pasteurization of milk and the campaigns of control and eradication of tuberculosis in cattle were implemented [75]. Contact with infected animals and ingestion of unpasteurized milk and dairy products, or incorrectly pasteurized, are the main routes of transmission of these zoonotic species [23, 75]. As mentioned above, in industrialized countries, human TB cases attributed to *M. bovis* were 0.5 to 7.2% in the last twenty years [23]. However, it is estimated that over 94% of the world's population live in countries where there are no strategies for the control of infections caused by *M. bovis* [76]. In these countries, many of which belonging to the African continent, bovine tuberculosis poses a significant threat to public health [1, 67, 70], where it is estimated to be responsible for 10 to 15% of new cases of human tuberculosis [23].

In Portugal, according to the World Health Organization (WHO), the last recorded cases of human tuberculosis caused by *M. bovis* occurred in 2003 and 2004 (Euro TB Reports, 1999-2006), but usually, only the *Mycobacterium tuberculosis* complex is identified in clinical laboratories. It was suggested that 3.4% of tuberculosis cases detected in *Hospital Amadora-Sintra*, between 1999 and 2002, were caused by strains of *M. bovis* [77], but subsequent studies have not confirmed the classification of these isolates [78, 79].

Tuberculosis is especially important in individuals with immunodeficiency, particularly in patients infected by human immunodeficiency virus (HIV), which are particularly susceptible to infection by mycobacteria [67, 70, 75]. The emergence of multi-resistant strains of *M. bovis* in humans [80], as is the case with *M. tuberculosis*, and the fact that transmission between humans can occur [21, 81], reveals worrying.

Human tuberculosis caused by *M. bovis*, from the clinical and pathological point, is indistinguishable of the tuberculosis caused by *M. tuberculosis*. Discrimination between these causative agents is possible only through laboratory diagnosis using conventional

and molecular methods. However, in the laboratorial diagnosis of human tuberculosis culture media are generally not suitable for isolation of *M. bovis* (needs to be supplemented with pyruvate – see 1.3.1) and, on the other hand, is carried out only the identification of the MTC, raising up the suspicion that *M. bovis* can be an undervalued cause of morbidity and mortality [67, 82]. This finding is particularly relevant in developing countries where, due to limited resources, the diagnosis is based only on viewing acid-fast bacilli (AFB) in smears [67].

1.2.2. Epidemiological features

Transmission of *Mycobacterium bovis*

The aerial route is the main and most important route of transmission of bovine tuberculosis [76], being facilitated by the high survival rate of *M. bovis* in aerosols [68]. The direct contagion by inhalation thus constitutes the most common route of infection. Another important way of transmitting the infection is by ingestion, which may occur through contaminated water or pastures, or even through infected replacer milk administered to calves [9]. The congenital infection and vertical transmission, as well as genital transmission, are rare, especially in regions where eradication plans operate [83, 84]. It is also uncommon the transcutaneous transmission, which may occur in situations of bitten by infected animals [85]. There is reference to the arthropods as vectors of infection, since viable *M. bovis* were detected in ticks, but this transmission path is very unlikely [69].

The infection by indirect way can occur since *M. bovis* have some resistance to the environmental conditions, being its survival dependant of factors such as temperature, humidity and sunlight [69, 86]. These mycobacteria can survive in the environment for a limited period (3 to 14 days) when exposed to sunlight, but can survive for a longer period (six weeks) under conditions of darkness and moisture. Eventually, mycobacteria can resist in fomites and faeces for two to six months [69, 86]. The survival of *M. bovis* in protozoa suggests that these organisms may provide protection against adverse environmental conditions [87], thus representing an environmental reservoir of *M. bovis* [88].

Global epidemiological situation

Bovine tuberculosis is a disease of worldwide geographical distribution (Figure 1.5), with high incidence in many developing countries, where it has great importance [1, 70]. Thanks to the implementation of eradication programs, several EU countries, including Austria, Denmark, Slovakia, Finland, Holland, Luxembourg, Czech Republic and Sweden, have managed to eradicate bovine tuberculosis, while other countries, including Portugal, only reduced the incidence of the disease (OIE, 2013).

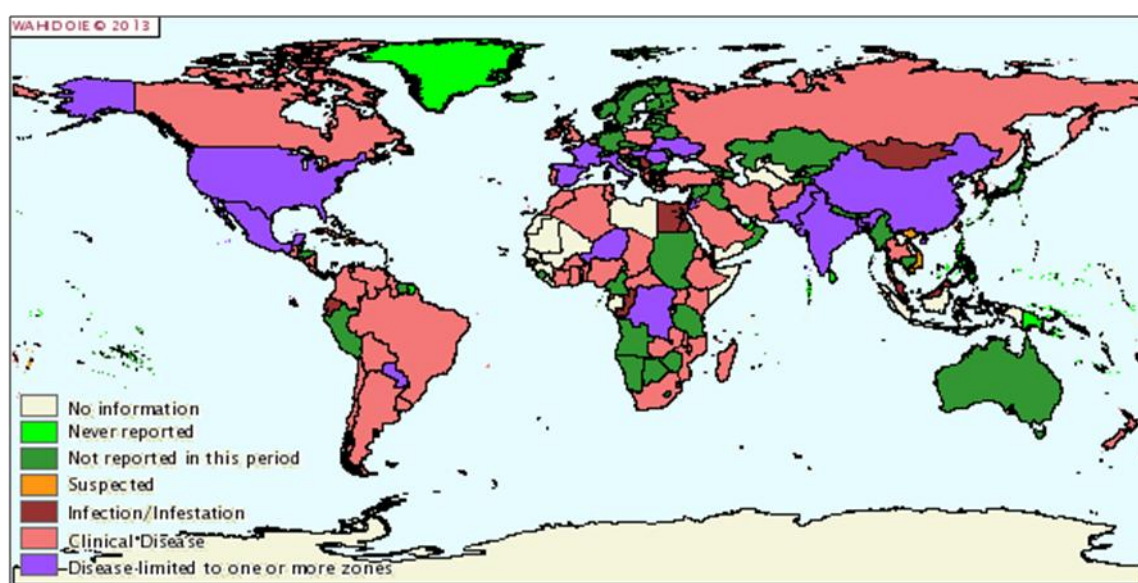


Figure 1.5. Worldwide distribution of Bovine tuberculosis (OIE, 2013).

In many countries, the eradication of bovine tuberculosis has been hampered by the presence of several wild animals which act as reservoirs in the maintenance of *M. bovis*, among which are the badger (*Meles meles*), the opossum (*Trichosurus vulpecula*), bison (*Bison bison*), the African buffalo (*Syncerus caffer*) and the white-tailed deer (*Odocoileus virginianus*) [89, 104, 229].

In Portugal, the plans for the control and eradication of bovine tuberculosis have been implemented since 1992, often with the financial support of the European Union. From 2000 to 2007, the prevalence of this disease in cattle decreased from 0.1% to 0.04%, lying now in the final and difficult stage of eradication of the disease [90]. To avoid the

potential risk of transmission it is important to use all the resources for the early detection of infected animals and herds, including serological and molecular *ante* and *post-mortem* laboratory diagnostic tests [143, 230]. The rapid detection of *M. bovis* is pivotal in contributing to the control and eradication of bovine tuberculosis.

Tuberculosis in free-ranging wildlife populations

Mycobacterium bovis and other MTC species have been recognized as a global threat at the wildlife-livestock-human interface. Several wildlife species have been identified as maintenance hosts. Spill over of infection from these species to livestock or other wildlife species may have economic and conservation implications, and potential infection of humans poses public health concerns. The existence of natural reservoirs (maintenance hosts) of the disease, capable of reintroducing it into farms free of tuberculosis, has complicated the control and eradication of bovine tuberculosis in many countries [76, 91].

The study of cases of tuberculosis in free-living fauna is especially difficult, which explains the difficulty in controlling this disease once established in these populations. The reasons most often cited for this are the limitations on the knowledge of own sylvatic populations, both in relation to the number of individuals and their identification (usually only relative abundances are known), or with regard to the understanding of its importance in the epidemiology of the disease [92, 93]. It is also recognized the difficulty of the detection of sick animals in sylvatic populations, since the symptoms are non-specific and are absent in most animals (the most common symptom is the weight loss which occurs in advanced stages of the disease). Furthermore, in many cases there are no plans of epidemiosurveillance, being during the hunting the only contact that man has to these animals. Thus, the detection of tuberculosis is based on the post-mortem diagnosis, through the observation of the characteristic lesions (very important for the hunted animal for consumption), supported by histological and bacteriological analyses. The appearance and distribution of the lesions can differ in different infected species, particularly between cattle and cervids [93, 94].

Although tuberculosis has been detected in various sylvatic species, does not mean that all these populations constitute a natural reservoir of the disease. Most of these species are accidental hosts, i.e., can acquire the disease, but if removing the source of infection, there is a reduction in the prevalence of tuberculosis, which is not maintained in the population [95]. On the other hand, there are species that are considered maintenance hosts, because the infection may persist in the population only by horizontal transmission. This ability to act as a reservoir and transmit the disease to other susceptible species depends on the prevalence of the disease in the population of the maintenance host, their behaviour and their relationships in the ecosystem [93, 96]. There are several examples of these sylvatic reservoirs of tuberculosis, responsible for the transmission of infection to domestic cattle, hampering the efforts to eradicate the disease in certain countries. Some of the most studied are the European badger (*Meles meles*) in the UK and Ireland and the possum (*Trichosurus vulpecula*) in New Zealand, countries where there was an observed decrease in the prevalence of the disease in domestic cattle after campaigns based on the elimination of infected populations of these sylvatic animals [96, 97].

In the state of Michigan, USA, the white-tailed deer (*Odocoileus virginianus*), also known as the Virginia deer or simply as the whitetail, is considered the responsible for the re-emergence of the disease in cattle, after bad options of management of this cinegetic species. Consequently, due to the practice of supplementary feeding in the winter, their number grew beyond the capacity of the ecosystem, encouraging behaviours that increase the risk of intra- and interspecific transmission. This maintenance host is still the source of outbreaks in several species of carnivores, such as the coyote (*Canis latrans*), raccoon (*Procyon lotor*), the black bear (*Ursus americanus*) or red fox (*Vulpes vulpes*), and a potential danger to the hunters [92]. The water buffalo or domestic Asian water buffalo (*Bubalus bubalis*) in Australia is an example of a sylvatic maintenance host associated with a well succeed eradication campaign of tuberculosis. This required the total slaughter of chronically infected herds and restocking with animals originating from a disease-free population [96, 97]. In Africa, the most studied natural reservoir of the disease is the African buffalo or Cape buffalo (*Syncerus caffer*), which has proved a source of spreading of the disease to the predators, particularly on natural parks of South Africa [95]. This situation raises

increasing concerns in the preservation of endangered species, including the larger felid species, such as lions (*Panthera leo*), cheetah (*Acinonyx jubatus*) and leopard (*Panthera pardus*) [98].

The first studies on tuberculosis in sylvatic species in the Iberian Peninsula were performed in Spain. Several studies have identified the wild boar (*Sus scrofa*) and the red deer (*Cervus elaphus*) as important reservoirs of the disease, reaching dispersed geographically populations and presenting a high prevalence of tuberculosis [95, 99, 100]. Although in certain European countries the wild boar is considered an accidental host, various molecular, anatomopathological and epidemiological studies have strengthened the role of the wild boar as a reservoir in Mediterranean ecosystems [101-103].

The population density of wild boar and deer has increased significantly, either by the lack of natural predators, or by the practices of cinegetic management of these species, such as the improvement of the hunting reserves fences (not relevant in the case of wild boar) and the food supplementation in the summer when the pastures are scarce [100]. In addition to the features mentioned above, also the behaviour of these animals and the evidence that they share *M. bovis* isolates with the same spoligotype profile as domestic cattle [95], make the wild boars and deer a risk factor in the reintroduction of the disease in disease-free farms. Also other domestic animals can be infected, including goats and Iberian pigs, in farms of extensive-type regime. These natural reservoirs of disease constitute a potential threat to public health (especially to the hunters and handlers of carcasses) and have also been implicated in transmission to endangered species such as the Iberian lynx (*Lynx pardinus*), endangering their preservation [100].

The control of tuberculosis in sylvatic wildlife is extremely complicated, an example is the partial control of populations of badgers done in some areas of the UK. These actions obtained counterproductive results, because the disruption of the normal social structure of badgers led to an increase of migration of infected animals, yielding a greater number of outbreaks of TB in cattle. On the other hand, the case of the Asian buffalo in Australia was the only example of a successful campaign for the eradication of this disease in a sylvatic maintenance host. In this case, the method used was the total

slaughter, but this is seldom adopted by economic, logistical, social and nature protection reasons [98].

In Portugal, the recognition of the presence of tuberculosis in wild boar and deer is longstanding [104, 229]. However, there is no national plan of surveillance of this disease in sylvatic species, however, exists only a few actions/studies in certain fields. Thus, it is not known the infection status of these populations. Currently, this information is summarized in some recent works about tuberculosis in wild boar [102, 104] and about the genotyping of strains responsible for tuberculosis in deer and wild boar [62, 105]. In Portugal, the prevalence of tuberculosis in cattle and the occurrence of the disease in wild boars are significantly associated, suggesting a relationship between the disease in these species [102]. Furthermore, according to this author, the presence of tuberculosis in wild boar is also significantly associated with the density and diversity of sylvatic ungulates. Therefore, the wild boar appears to be the maintenance host of *M. bovis* in Portugal, and satisfies the theoretical conditions for being regarded as the reservoir of tuberculosis in our country [102]. Nevertheless, the great diversity of patterns found in isolates of deer from the same geographical area suggests that these animals are accidental hosts and not reservoirs of the disease. However, there is strong evidences of transmission of *M. bovis* between cattle and deer or wild boar, although not fully understood the meaning of it [62].

1.3. Diagnosis of bovine tuberculosis

In cattle, symptoms of tuberculosis often manifest later, namely by the presence of lesions in tissues, so the clinical diagnosis is rare, especially in the course of control and eradication plans [23]. In this context, the screening of tuberculosis in live animals (*in vivo* or *ante-mortem* diagnosis) depends on immunological diagnostic tests, such as the single intradermal cervical tuberculin test (SICCT) and the interferon-gamma (IFN- γ) test, which are based on the detection of cell-mediated immune responses. The use of these tests represents an important step for the eradication and monitoring of the disease. The confirmation of the presence of infection and consequently the definitive diagnosis of bovine tuberculosis, is made *post-mortem* usually based on anatomopathological and histopathological diagnosis, on the culture and isolation of *M.*

bovis in suspicious lesions, and through molecular analysis to confirm the presence and the identification of the etiological agent. Since bovine tuberculosis manifests itself as a public health problem, it is an international research priority the development and improvement of new methodologies for a more effective diagnosis of this disease, whether conventional or molecular based. A rapid and specific laboratory identification of the etiologic agent of this disease appears extremely important, contributing to a better understanding of its epidemiology and leading, ultimately, to a more effective control of the infection in cattle and the improvement of the plans for the eradication of bTB.

1.3.1. Conventional methodologies

Immunologically-based approaches

The immunological diagnostic tests are fundamentally based on cell-mediated immunity, since the cell-mediated response is a major defence mechanisms to infection by *M. bovis* [23]. Only in the later stages of the disease are produced antibodies in measurable quantities [84, 106, 107], whereby the diagnostic methods based on the humoral response have, in general, low sensitivity for early diagnosis than those that are based on cell-mediated response [108, 230].

The single intradermal cervical tuberculin test, approved by the European Union, is used in Portugal for the diagnosis of tuberculosis in cattle, and is also the reference test advocated by the World Organization for Animal Health (OIE) for the international marketing of cattle [23]. This test is based on the observed delayed hypersensitivity reaction, after intradermal injection of bovine and avian tuberculin, in animals who contact with *M. bovis* (vaccine strain or other). Tuberculins are water-soluble purified protein derivatives (PPD), obtained from the growth of certain strains of *M. bovis* (PPD-B) and *M. avium* (PPD-A), namely the AN5 and D4ER strains, respectively, in defined culture medium. As with any diagnostic test, there are several factors that can give a false-positive or false-negative result with SICCT tests. The first case includes, for example, the infections caused by mycobacteria not belonging to the MTC and, in the second case, the energy developed in cattle with advanced or generalized tuberculosis.

It is estimated that the sensitivity of SICCT test ranges from 75% to 95.5%, with a higher specificity between 78.8% and 100%, due to the use of the two tuberculin [23].

The IFN- γ test is an enzyme immunoassay (ELISA) based on the evaluation of cytokine released by lymphocytes previously sensitized, with bovine and avian tuberculin [23]. Its sensitivity is high, but has a lower specificity than the SICCT assay. Generally the IFN- γ test is used as a complement of the SICCT test, allowing the early detection of infection because of the extreme sensitivity that results from the combination of the two tests [109]. In Portugal, this test is recommended in cases of doubtful animals to the SICCT test or other situations defined by the National Plan for the Eradication of Bovine Tuberculosis [72].

In addition to the IFN- γ test, there are other laboratory assays based on cellular immunity, namely the lymphocyte proliferation and, also, several tests based on humoral immunity, namely serological tests. Due to the specialized technical implementation and costs involved in its realization, all these tests are presented as additional to the SICCT test.

As mentioned above, most immunological diagnostic tests for bTB uses the tuberculin as reagent, which consist of a mixture of peptides with different antigenic properties. Many of the false-positive reactions of the cattle to the intradermal inoculation are due to the antigenic complexity of the PPD [110]. The characterization of the antigens of *M. bovis* has allowed the development of novel diagnostic tests with higher specificity and sensitivity. Several antigens of *M. bovis* have been studied in the last years, including the ESAT-6 and CFP-10 proteins, which have proved useful for the detection of infection, being a complement, or even an alternative to the tuberculin [110-115]. These antigens are capable of stimulating whether the cell-mediated response or the humoral response, and may therefore be used in various immunological tests *in vitro* and *in vivo* with various objectives, including the discrimination between infected and vaccinated animals [116, 117].

Anatomopathological diagnosis

The observation of typical macroscopic lesions (tubercles) on *post-mortem* examination, in conjunction with histopathology, allows the presumptive diagnosis of bovine

tuberculosis [118]. The lesions are often found in the lungs and retropharyngeal, mediastinal and bronchial lymph nodes, although they may appear in other organs [119]. In microscopic observation, the granulomatous lesion is characterized by a central caseous necrosis, sometimes with calcification, surrounded by epithelioid cells, multinucleated giant cells, macrophages and lymphocytes, being encapsulated by fibrous conjunctive tissue [120].

The presence of acid-fast bacilli (AFB) in the lesions, revealed in histological sections subjected to the specific staining of Ziehl-Neelsen or fluorescent staining with auramine, corroborates the presumptive diagnosis of tuberculosis. This technique, while rapid and inexpensive, can only show the presence of AFB cells in concentrations higher than 10^4 bacteria per ml and the procedure also does not allow the distinction between the members of the family *Mycobacteriaceae* [121]. It was also shown that the Ziehl-Neelsen staining is affected by changes in the cell wall of *M. tuberculosis* during the latency states [122]. Alternatively, the detection and identification of MTC bacteria, in histological sections, could be achieved by immunohistochemical techniques using antibodies specific for the complex, making it a more sensitive and specific procedure [122-124]. In addition to this technique, also molecular techniques, including *in situ* hybridization, have been used in histological sections for the detection and identification of MTC species [125].

Although the microscopic observation of acid-fast bacilli in smears corresponds to tuberculosis in most cases, an efficient diagnosis often difficult due to the poor sensitivity (e.g. in paucibacillary lesions in cattle) and specificity of the assays, being imperative the use of culture-based methods to detect the presence of the etiological agent.

Bacteriological diagnosis

Due to the nature of MTC mycobacteria, bacteriological diagnosis of tuberculosis involves biosecurity measures of containment level 3, with very demanding operational requirements and physical facilities. This biosafety level limits the number of laboratories with conditions able to perform this type of diagnosis.

The isolation of *M. bovis* from biological samples, including macerated tissues of animals suspected of having tuberculosis, is considered the most effective means for the definitive diagnosis of bovine tuberculosis. This method has high specificity, but is not particularly sensitive, especially when the culture is obtained from lymph node samples of an animal without observable lesions, albeit positive for SICCT or IFN- γ test [23, 84]. The successful isolation of *M. bovis* is also influenced by the decontamination procedures of the samples, the culture medium used, and the incubation conditions of the cultures [118]. The viability of mycobacteria can be affected by the action of the reagents used in the decontamination procedures, necessary for the elimination of rapidly growing microorganisms existing in the samples. There are several decontamination methods available, suitable for each type of biological sample, all being based on the use of acids, alkalis or detergents. For animal tissues, the most recommended decontamination reagents are sodium hydroxide at a final concentration of 2% and the hexadecyl pyridine chloride at 0.075% [126]. Several antimicrobial substances have also been used to inhibit the growth of contaminating microorganisms, to which the mycobacteria are resistant, such as penicillin derivatives, trimethoprim, nalidixic acid, amphotericin B and cycloheximide. Traditionally, the most used selective culture media for MTC species are the Lowenstein-Jensen supplemented with pyruvate, the Stonebrink and the Middlebrook 7H11 [127]. In these solid media, the growth of *M. bovis* is slow due to the long generation times (16-20h) of this bacteria, and it takes three to eight weeks until colonies are visible. The introduction of automatic radiometric (e.g. BACTEC 460) and fluorometric (e.g. BACTEC 9000MB or MGIT 960) equipment contributed to shorten the culture time for between two to three weeks [82, 128].

The identification of the mycobacterial isolates, based on the analysis of phenotypic traits, is carried out by assessing the colony morphology, the production of pigments and a set of biochemical tests, such as the susceptibility to the presence of TCH (Thiophene-2-carboxylic acid hydrazide), the production of niacin and the reduction of nitrate [129]. These identification procedures are also time consuming (three to four weeks), and sometimes are non-conclusive, for example, in the case of strains of *M. bovis* with features common to *M. tuberculosis* [130]. The bacteriological diagnosis of tuberculosis is thus an extremely lengthy procedure (eight to ten weeks), and not always

allowing the discrimination of the MTC species. The species identification may also be achieved by the analysis of mycolic acids by high-performance liquid chromatography (HPLC) [131]. This technique is, however, sophisticated and requires very expensive equipment and highly skilled technicians. The identification of mycobacteria obtained by the isolation in culture can be also done using molecular methods.

1.3.2. Rationale of the molecular-based strategies for the assessment of the MTC

Among the most promising segments of the *In Vitro* Diagnostics (IVD) market, with growing rates of 20% per year, is the molecular diagnosis, mainly based on the analysis of nucleic acids (80% of this segment corresponds to the diagnosis of infectious diseases). The molecular diagnosis technologies are usually associated with improvements in sensitivity, specificity and time response comparing to other conventionally methods that are used. The market trend is the increasing substitution of the conventional methods by the molecular ones, with some emphasis for the rapid tests.

Detection and identification

DNA amplification-based assays

Rapid diagnosis reports to a wide range of novel testing procedures which can significantly reduce the reporting time within hours or even minutes compared with that of conventional culturing methods. Diagnostic tests should ideally meet certain requirements, they must be cheap, robust and simple enough to be used by minimally trained health technicians. Technological innovations are emerging that could address many of the unmet diagnostic needs for diseases like tuberculosis. In this context, various nucleic acid-based techniques have been developed, including Polymerase Chain Reaction (PCR), Nested PCR, Real-time PCR, Loop-mediated isothermal amplification (LAMP), and sequence-based identification, among others. The molecular methods seems promising for a faster and specific diagnosis of tuberculosis, however, it is necessary to make them simpler, standardized and accessible to a wider and effective use in the routine clinical laboratory.

There are several PCR protocols to enhance detection sensitivities, especially when dealing with small numbers of bacterial cells as target. Nested PCR is one of these approaches, allowing the detection of only a few bacteria in clinical specimens [132]. The process utilizes two consecutive PCRs. The first PCR contains an external pair of primers, while the second contains either two nested primers that are internal to the first primer pair or one of the first primer and a single nested primer. The larger fragment produced by the first reaction is used as the template for the second PCR [132]. The sensitivity and specificity of DNA amplification can be considerably improved by using such nested PCR strategies, sometimes with one thousand times more sensitivity than a standard PCR.

Most conventional first-generation PCR assays involved complex procedures for detecting the amplification products (e.g. gel electrophoresis and ethidium bromide staining). A great technological advance towards the simplification of nucleic acid testing assays was the development of quantitative real-time PCR, which involves the amplification and detection of amplified products coupled in a single reaction vessel, increasing the speed of detection [133]. This allows for the direct detection of the PCR product during the exponential phase of the amplification reaction, combining amplification and detection in one step. The measurement of the rate of increase of the amplification products in real-time also offers the possibility of extrapolating back to the starting DNA concentration, thus offering the possibility of quantification [134]. The ability to monitor the progress of DNA amplification in real-time depends on the chemistry of the reaction and instrumentation used. Generally, chemistries consist of special fluorescent probes that must associate a fluorescent signal to the amplification of DNA [135]. The initial real-time PCR procedures made use of the dsDNA-specific intercalating dye ethidium bromide [136]. dsDNA-specific dyes exhibit little or no fluorescence when free in solution, but produce a strong fluorescence signal when bound to dsDNA and exposed to the appropriate wavelength of light. Although the use of other dyes has been described, SYBR Green I [137] is currently the industry standard [135]. Intercalating dyes are the most cost effective chemistry and usually are the first choice for many applications in research and diagnostics. A disadvantage of using these dyes is that any double stranded DNA will be detected, e.g. non-specific amplicons and primer dimers, which may cause problems with the accuracy of quantitative PCR.

Because of this limitation, other strategies that involve the use of probes that will specifically recognize only the target PCR product have been developed. The most common probes currently used in real-time PCR assays are the *Taqman* probes, the molecular beacons and fluorescence resonance energy transfer (FRET) probes [138].

Taqman probes are one of the most widely used chemistries because the assay design is relatively simple and generally robust. These probes have a 5'-end fluorescent reporter molecule and a 3'-end quencher molecule (Figure 1.6). The fluorescence of the reporter molecule is quenched when in the proximity of the quencher molecules. The hydrolysis of the probe by the 5' exonuclease activity of DNA polymerases separates the reporter and quencher molecules and a fluorescent signal is emitted and detected by the real-time PCR instrument. The incubation temperature of the DNA polymerization step is critical since at higher temperatures (e.g. 72 °C), which are normally used for DNA synthesis in conventional PCR, the polymerases will displace the probe rather than degrade it. As a result of probe degradation, the fluorescent signal increases as a function of the number of amplification cycles and allows the specific detection and quantification of the target DNA. Molecular beacons use secondary structure (stem-loops) to hold both the reporter and quencher molecules in close proximity when the probe is in solution, preventing the production of any fluorescent signal [139]. When the probe anneals to the target DNA, they unfold and the distance between the reporter and quencher molecules is sufficient to allow the emission of fluorescence. These probes can be used for performing DNA melting curve analysis (by measuring the dissociation kinetics of the release of the bound probe to the target DNA), allowing a further genetic characterisation of the amplified DNA. Fluorescence resonance energy transfer (FRET)-based assays rely on the energy transfer between a 3'-end donor fluorophore and a 5'-end reporter fluorophore on separate probes, rather than the quenching of a fluorophore as seen with molecular beacons and *Taqman* probes [135, 140]. Melt curve analysis in FRET assays measures the temperature at which the bound probes are dissociated from the target amplicon and not the melting temperature of the entire amplicon as is the case when using intercalating dyes. Thus a single primer set may be used to amplify a region of interest, with species/strain discrimination possible by designing the FRET probes to bind to variable regions within the region bound by the primer set used for amplification. This technology lends itself to SNPs detection, as a single mismatch

between the probe and target sequences will yield a sufficiently different melting temperature to allow detection.

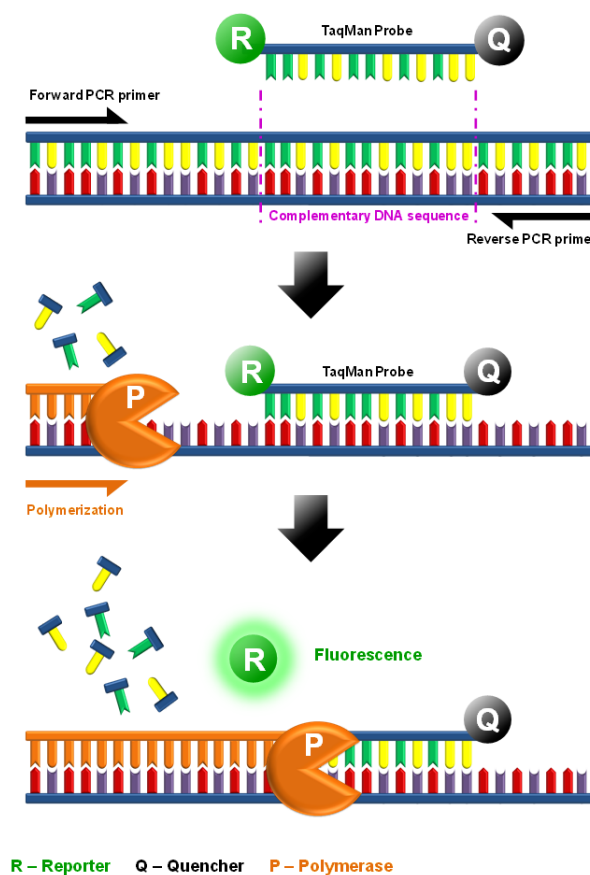


Figure 1.6. Schematic representation of *TaqMan* chemistry (original drawing by Pedro Costa).

Over the past years, numerous methods have been described for the molecular detection and identification of mycobacteria, including MTC members, using for example nested PCR approaches [141-143] and several real-time amplification chemistries [143-150]. Some molecular assays are also commercially available, such as AccuProbe (Gen-Probe Inc., San Diego, USA), Inno-LiPA (Innogenetics, Ghent, Belgium) and GenoType Mycobacteria (Hain Diagnostics, Nerhen, Germany). The detection and identification of *M. bovis* directly in samples suspected of bovine tuberculosis have been achieved by several authors by using various DNA amplification-based techniques [151-157], which

showed varying sensitivities, generally low. The characteristics of the lesions (usually with fibrosis and calcification) associated with the presence of few bacilli (paucibacillary samples) and inhibitors are responsible for the difficulties encountered in the direct identification of *M. bovis*. The nucleic acid extraction from mycobacterial cells is also difficult, associated with the characteristics of the cell wall. Although various strategies have been developed to overcome this problem, in particular through sequence-capture techniques of specific DNA sequences [154, 155], nested-PCR [153] or multiplex real-time PCR [156], the sensitivity of these methods is usually lower, in comparison with the bacterial culture, rarely exceeding 75%. Other molecular methods, including *in situ* hybridization techniques, were used for the detection and identification of MTC members in tissues [125].

The discrimination between species of the MTC is more difficult to achieve due to the high genetic homology that exists between them. Sequencing of the genomes of *M. tuberculosis*, *M. bovis* and *M. bovis* BCG, coupled with the advances of bioinformatics, has contributed to the identification of genomic regions useful in the discrimination of the species of the MTC. The analysis of the polymorphisms of the *gyrB* gene by PCR-RFLP allows the differentiation between the main members of the complex, with the exception of *M. tuberculosis* and *M. africanum* type II [158]. The analysis of the *gyrB* gene polymorphisms have also been used for other bacterial genera, for the species differentiation and inferring phylogenies. Another molecular approach is the analysis of Regions of Difference in the genome of these species [3, 43]. As already mentioned above, the pattern of presence or absence of certain RDs appear to reflect its evolutionary history, so their respective analysis is presented as a reliable alternative for proper identification of these species [147, 159, 160]. Also the differentiation between virulent and vaccine (BCG) strains is possible through the analysis of the RDs. There is an absence of RD1 in BCG strains, where genes involved in the production of virulence factors are located (for example, the ESAT6 protein). Mishra and colleagues (2005) reported another molecular approach, based on the analysis of *hupB* gene by the technique of nested-PCR, for the direct detection and identification of *M. tuberculosis* and *M. bovis* in biological samples of animal origin. Several genomic regions have been used as a target for the detection and identification of members of the MTC. For example, the analysis of the *mtp40* gene, which encodes a phospholipase C, was used to

differentiate *M. tuberculosis* and *M. bovis*, this gene being absent in the second species. However, it has been discovered that this gene is also absent in some strains of *M. tuberculosis* [8]. The *oxyR* pseudogene and *pncA* gene, which encodes for the pyrazinamidase [161-163], have, each one, a single base polymorphism between *M. bovis* and *M. tuberculosis*, but do not allow the differentiation of other members of the complex, such as *M. africanum* [8].

Isothermal DNA amplification

Novel developments in molecular diagnostic tools have demonstrated the possibility of nucleic acids amplification under isothermal conditions, i.e. without thermal cycling. A most promising method termed Loop-Mediated Isothermal Amplification (LAMP) was described that can amplify DNA or RNA with high specificity, efficiency and rapidity under isothermal conditions [164]. Unlike PCR, a denatured template is not required [165] and DNA is generated in large amounts in a short time. Furthermore, positive LAMP reactions can be assessed with the naked eye [166, 167]. The main advantage of this technique is its simplicity; only a water bath or heating block is needed to provide a constant temperature as the amplification proceeds under isothermal conditions.

The LAMP method employs a DNA polymerase and a set of four specially constructed primers that recognize six distinct sequences on the target DNA. An inner primer pair with sequences of sense and anti-sense strands of the target template initiates LAMP. A pair of outer primers then displaces the amplified strand with the help of a DNA polymerase which has a high displacement activity, to release a single stranded DNA, which then forms a hairpin to initiate the starting loop for cyclic amplification. Amplification proceeds in cyclical order, each strand being displaced during elongation with the addition of new loops with every cycle. The final products are stem loop DNAs with several inverted repeats of the target and cauliflower-like structures with multiple loops due to hybridization between alternately inverted repeats in the same strand [164] (Figure 1.7). The reaction can be accelerated by using two extra loop primers. There are several works reporting the use of this technology to detect pathogenic organisms, including mycobacteria [168-173].

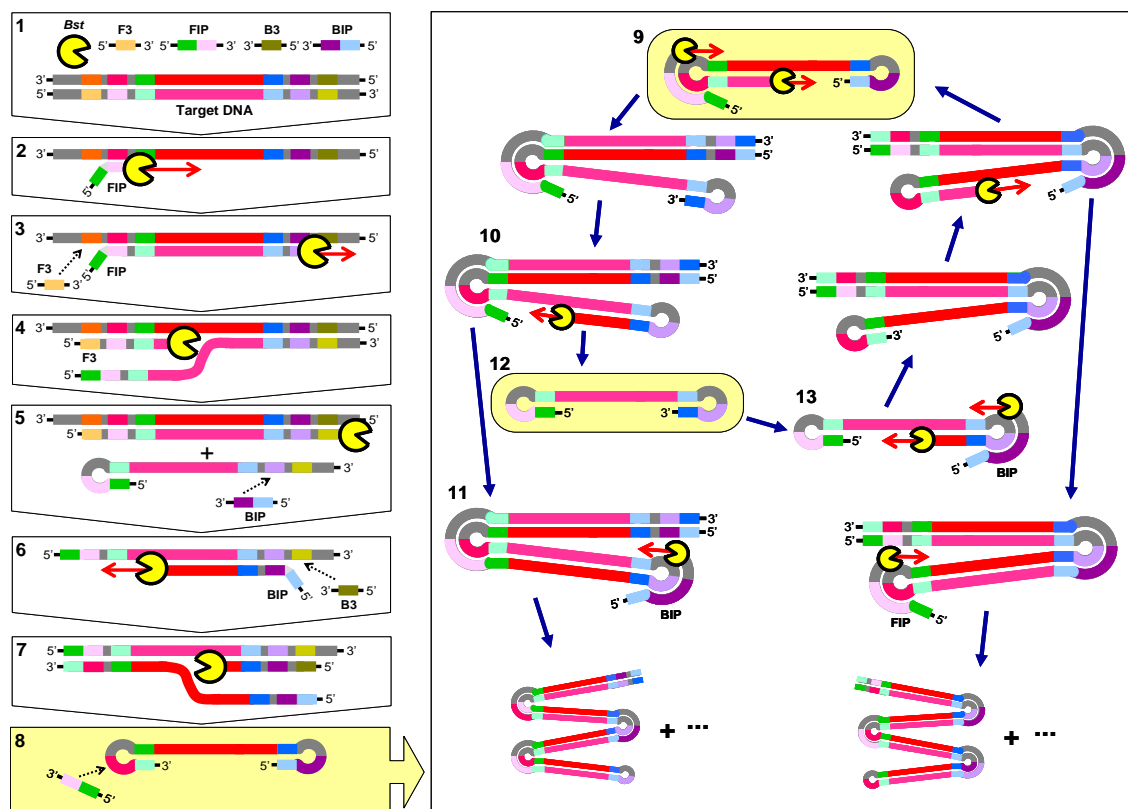


Figure 1.7. Schematic representation of Loop-mediated isothermal amplification assay (original drawing by Pedro Costa). LAMP is simple and requires only basic laboratory equipment, being characterized by the use of four primers (F3, B3, FIP and BIP), in an isothermal (60 – 65 °C) auto-cycling strand displacement reaction. FIP and BIP primers contain two distinct sequences corresponding to sense and antisense segments of the target DNA, one for priming in the first stage and the other for self-priming in subsequent reaction steps. Amplification products are a mixture of stem-loop DNA structures of various lengths containing several inverted repeats of the target.

Immunochromatographic assays

Immunochromatographic assays were first described in the late 1960s and were originally developed to assess the presence of serum proteins [174, 175]. However, over the past decade many other applications have been developed for immunochromatographic assays, including the detection of bacterial pathogens [176-180].

Typical handheld assay devices contain a colloidal gold (or other)-labelled antibody dried onto a filter pad affixed to a nitrocellulose strip. A capture antibody is applied in a line on the strip and dried. To perform the test, a specimen is suspended and added to the pad containing the colloidal gold-labelled antibody. At a critical point of interface, clumping of the carrier particles occurs as an indicator of the antigen-antibody interactions occurring on the surface of the carriers. A positive reaction is visualized as a red line created by the bound colloidal gold. Similar assays using different detection systems have been described in the literature, including those based on latex particles [175]. The increasing demand for point-of-care (POC) (or point-of-decision) diagnostics, led to the development of rapid tests to be used directly in the field. Most of these rapid tests rely in the format of dipstick tests, which are based on immunochromatographic assays (Figure 1.8). The dipstick tests provide qualitative (or semi-quantitative), fast, economical and easy to interpret results, the reading is usually taken with the naked eye. Dipstick assays enable endpoint nucleic acid detection. In recent decades, dipsticks-based assays were developed for the detection of several infectious diseases such as dengue fever [181], malaria [182], hepatitis B [183], among others. Assays using this format are rapid, taking approximately 15 min to run, and are also user-friendly. These tests are, therefore, of great value in situations where health professionals need to make decisions and take immediate measures. Dipstick-based assays were also previously described for the diagnosis of tuberculosis [184-186].

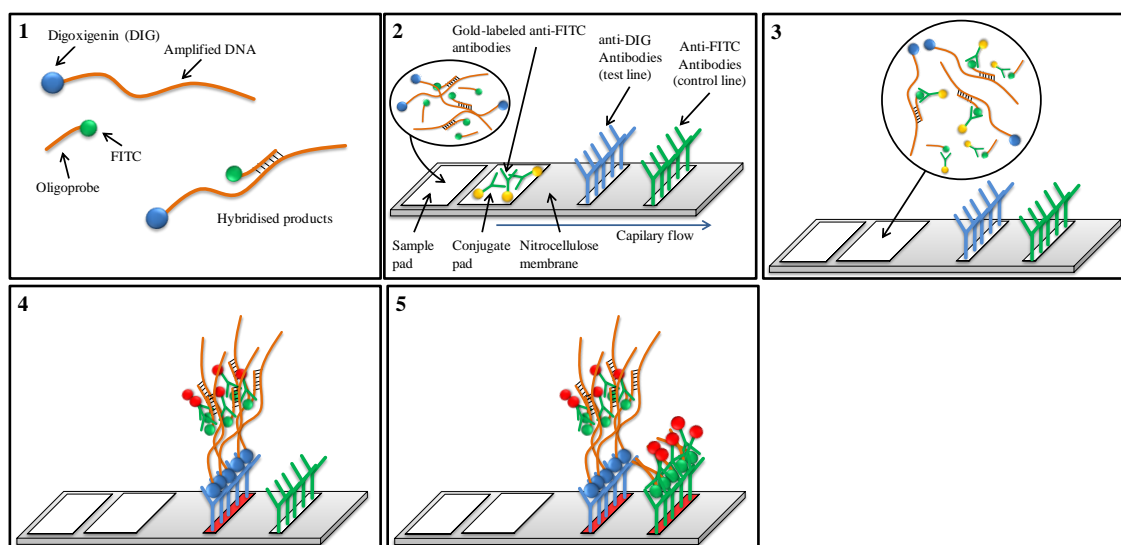


Figure 1.8. Schematic representation of immunochromatographic dipstick assay (original drawing by Pedro Costa). A common architecture involves the following steps: **(1)** Hybridization of DIG-labeled amplified DNAs with complementary FITC-labeled oligonucleotide probes; **(2)** Deposition of the hybridized products in the sample pad (the mixture also contains non-hybridised oligoprobes); **(3)** After diffusion of the hybridized products in the dipstick, a complex with gold-labeled anti-FITC antibodies is generated in the conjugate pad; **(4)** The resulting triple complexes migrate through the membrane and pass the test line, where the DIG molecules are captured by anti-DIG antibodies and a red test band, resulting from the concentration of gold nano-particles; **(5)** Non-hybridized FITC-labeled probe is bound by the gold-labeled anti-FITC antibody to form a double complex without DIG and moves through the test line to be trapped at the control line, containing anti-FITC antibodies.

Nanotechnology and nanodiagnosis

The growing interest in increasingly miniaturized materials and devices led to the emergence of a new technology field, named nanotechnology [187]. This new branch can thus be defined as the technology capable to produce and implement structures, machines, devices and systems, with controlled shape and size in a nanometre scale and the exploitation of new properties and phenomena that develop at this scale. The concept of bionanotechnology, which includes the nanodiagnosis, arises from the development of new methodologies and new platforms at nanoscale with biological application, in particular, for the development of biosensors, nanodiagnosis of infectious diseases, nucleic acid detection, detection of SNPs, "drug delivery", gene therapy, and

other applications [187]. This technology is now being applied for field detection combining microfluidics, nanoprobe, paper based platforms and user-friendly detection procedures for the development of low-cost point-of-care diagnosing testing for infectious diseases such as TB and leishmaniasis [231, 232].

The use of nanoparticles (NPs) for the development of molecular diagnostic methods is promising, particularly the nanoparticles of noble metals such as gold [188, 189]. NPs have a size between 1 and 500 nm and can be found dispersed in solid, liquid or gaseous medium. Most of them has a spherical shape, with specific physical and chemical properties that allows their detection, analyses and quantification in a more efficient manner. The metal NPs can be formed by various noble metals, such as gold (Au), platinum (Pt) and silver (Ag). Of all the metal nanoparticles, gold nanoparticles (AuNPs) are of the most interesting and led to an intensive study of their optical, magnetic and electronic properties. When excited by an incident electromagnetic field, these nanoparticles produce an effect known as surface plasmon resonance (SPR), which is the collective oscillation of free electrons on the particle surface, producing properties of intense absorption and electric scattering. SPR depends on the particle size, which is comprised of the material and the shape. The surface plasmon resonance of the gold nanoparticles is responsible for their intense colours. In solution, monodisperse gold nanoparticles show a red colour, with a peak of the SPR band in the region of 520 nm. Conversely, a solution containing aggregated gold nanoparticles shows a blue colour, corresponding to a red shift of the SPR band peak to approximately 600 nm. These nanoparticles can be functionalized with thiolated oligonucleotides [190], yielding the so called gold nanoprobe (Au-nanoprobe). In 1996, Mirkin and co-workers first described a method of colorimetric detection of DNA based on gold nanoprobe [191]. The method consists of using single-stranded DNA oligonucleotides that can be detected through two different nanoprobe, each one functionalized with an oligonucleotide complementary to one of the target regions. The hybridization of the target with the two nanoprobe results in the formation of a polymer network (cross-linking method) which promotes the approach of the gold nanoparticles enough to result in a change of solution colour from red to blue.

An alternative strategy uses a single probe which hybridizes to target DNA fragments, and an increase in the ionic strength of the solution causes the aggregation of the

nanoparticles, causing the red-shift of the SPR peak (blue solution). The presence in solution of nucleic acids with a sequence complementary to the nanoprobe stabilizes the system and the solution maintains the initial red colour. The absence of a complementary sequence does not prevent the aggregation of the nanoparticles and the solution changes the colour to blue, visually observable, and can be confirmed by visible spectrophotometry (Figure 1.9). This colorimetric detection approach, whose gold nanoprobe aggregation is induced by increasing the salt concentration in the solution, is called of non-cross-linking method and has been used for the detection of specific sequences of DNA and RNA, including the characterization of mutations and single nucleotide polymorphisms (SNPs) [192-194, 233], the study of gene expression [195, 196] and the detection and identification of pathogens, particularly MTC members [194, 197].

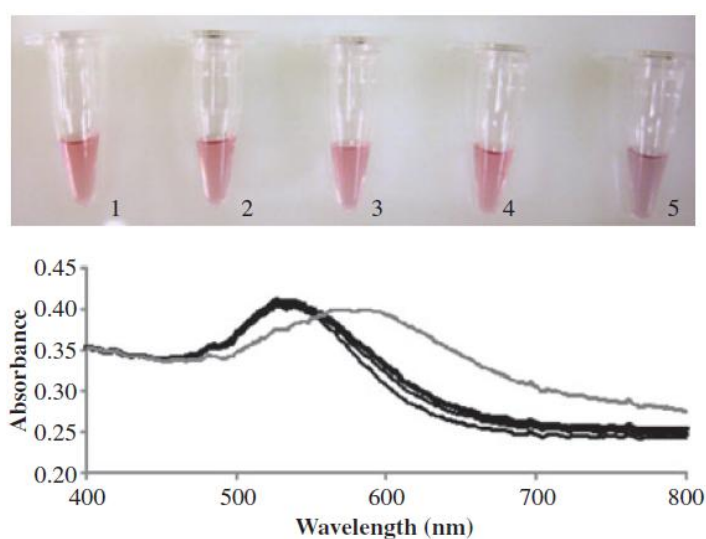


Figure 1.9. Gold nanoprobe assay. Colorimetric assay (above) and respective spectrophotometry (below) for the *Mycobacterium tuberculosis* complex (MTC) probe (black lines represent the red solutions associated with complementary target: non-aggregated Au-nanoprobes; the grey line represents the blue solution associated with non-complementary target: aggregated Au-nanoprobe) [194].

Characterization and genotyping

Genotyping technologies allow the characterization of different strains of the same species, using molecular biology approaches that analyse a part or the entire genome of the microorganisms. An efficient molecular typing assay should have a high discriminatory power in order to allow a clear differentiation of a large number of strains and possess high reproducibility. In the case of bovine tuberculosis, genotyping is a very important tool in determining the source of an outbreak and in identifying dominant epidemiologic patterns in different countries or geographical regions, to clarify the taxonomy of the species and subspecies and to elucidate possible associations between genotype and pathogenicity/virulence of different strains [198-204]. A precise and depth knowledge on the epidemiology of bovine tuberculosis, including an understanding of the disease transmission between cattle and the role played by other domestic and wild species in the maintenance of the disease, is essential to achieve the control and ultimately the eradication of the disease.

Currently, the genotyping techniques most used to characterize MTC isolates, in particular *M. bovis*, are the so-called Spoligotyping (Spacer Oligonucleotide Typing) and MIRU-VNTR (Mycobacterial Interspersed Repetitive Unit-Variable Number Tandem Repeats) analysis. These techniques are based on the amplification, by PCR, of specific polymorphic regions of the genome. Usually these regions have in common the presence of repeated nucleotide sequences, constituting ideal targets for *in vitro* amplification. These PCR-based techniques have the advantage of not requiring purification of template DNA and may be directly used in clinical samples [205], on stained smears [206] and even in archaeological specimens whose DNA is damaged [207-209]. These methodologies have been used in most laboratories where it is done the typing of *M. bovis*, because they are relatively simple, rapid and inexpensive [80].

Spoligotyping

Spoligotyping, whose name is derived from Spacer Oligonucleotide Typing, is based on the analysis of the polymorphisms of the DR *locus* [210], present in the genome of MTC mycobacteria. The DR region is composed by direct variable repeats (DVR). Each one of these DVRs is formed by a repeated sequence of 36 base pairs (DR) and the

adjacent variable spacer sequence, which has between 34 and 41 base pairs [210-212] (Figure 1.10). The order of the spacer sequences in the DR region is conserved [213] and polymorphisms are related to the number of direct repeats and the presence or absence of specific spacer sequences, with a total of 43 [40, 204, 214].

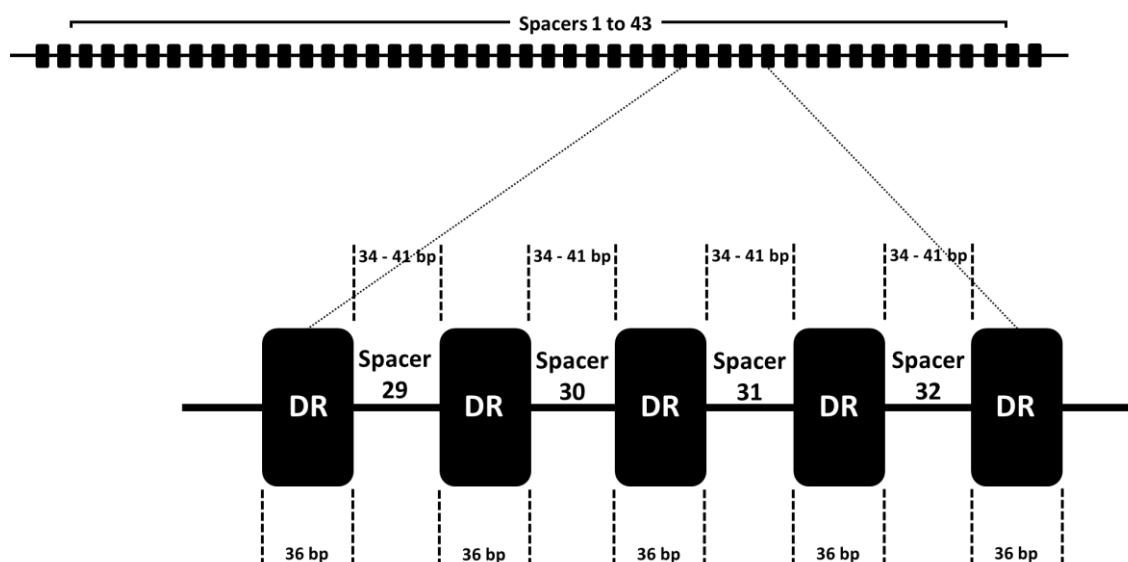
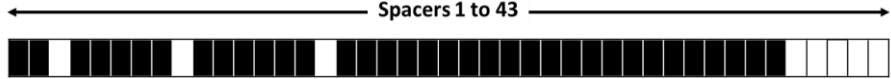



Figure 1.10. Schematic representation of the structure of the DR locus in the mycobacterial genome, constituted by conserved direct repeats, with 36 bp (rectangles), interspersed by variable repeat sequences, with 34-41 bp (continuous lines). DR - Direct Repeat; 1 to 43 – Spacers that may exist in a given strain (original drawing by Pedro Costa).

Spoligotyping applies to all members of the MTC. Some species and subgroups of strains may show characteristic spoligotyping profiles, known as signature profiles [215] (Table 1.4). For example, strains of *M. bovis* are characterized by the absence of spacer sequences 3, 9, 16, and 39 to 43 [62, 210, 213], while strains of *M. caprae* are characterized by the absence of the spacer sequences 1, 3 to 16, 28 and 39 to 43 [215, 216].

Table 1.4. Spoligotyping profiles characteristic of *M. bovis* and *M. caprae*.

MTC species	Spoligotyping signature ¹
<i>M. bovis</i>	
<i>M. caprae</i>	

¹ The black squares represent the presence and the white ones the absence of each one of the 43 spacer sequences (original drawing by Pedro Costa).

Experimentally, spoligotyping involves the extraction of genomic DNA from the mycobacterial isolates and the amplification of the DR locus by PCR, which is then used for reverse hybridization in a membrane where DR-specific complementary oligonucleotides are immobilized at specific locations. After the revelation of the occurrence of hybridization events, each of the present spacers will produce a dark spot, while the absence of a specific spacer is indicated by the absence of the dark spot in the respective position. The spoligotyping profile can be transformed into a binary code (0 represents the absence of the spacer sequence and 1 the presence of the same one), making it possible to compare the obtained profile with other existing in international databases such as Mbovis.org (Figure 1.11). In this database, each profile is denoted by SB (Spoligotype Bovis) followed by four numbers, like for example SB1174.

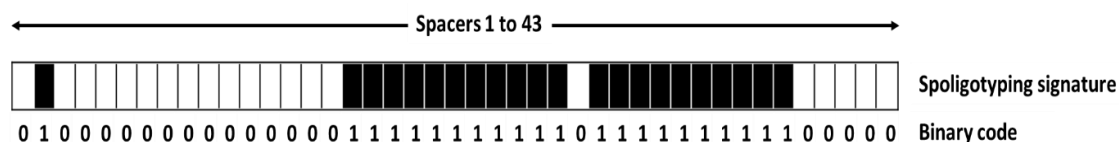


Figure 1.11. Illustration of a spoligotype profile (*M. caprae*) and its correspondent binary code. For each spacer the dark band and number 1 indicates the presence of the spacer; the absence of the band and a 0 number indicates the absence of the spacer (original drawing by Pedro Costa).

Although the strains of a same MTC species usually lack certain characteristic spacer sequences, they may differ intra-specifically in the presence or absence of other spacer sequences, and thus may yield specific spoligotype profiles. Therefore, the spoligotyping method allows the simultaneous detection and typing of MTC strains [210]. However, despite spoligotyping is regarded as the technique of choice, in a first approach, for the genotyping of *M. bovis* isolates [63, 198], due to its good degree of reproducibility and discrimination, the discriminatory power is smaller than the MIRU-VNTR technique [80].

MIRU-VNTR

MIRU-VNTR analysis is based on the investigation of multiple loci in the genome of the organisms, which have a variable number of tandem repeats (VNTRs). In the genome of MTC members, these tandem repeats are referred as MIRUs (Mycobacterial Interspersed Repetitive Units) and are sequences with approximately 40 to 100 base pairs dispersed in certain intergenic regions [217-220]. The molecular typing by MIRU-VNTR allows the detection of the existent polymorphisms between different strains, namely the hyper-variability on the number of MIRUs present in some of the loci analysed. This technique has the advantage that it can be automated, by PCR amplification, using primers labelled with fluorophores, making it possible to determine the number of tandem repeats for each locus under study, based on the size of the amplified products [221, 222].

The polymorphism of each locus is variable, and the number of loci studied depends on the MTC species and the purpose of the study (e.g. phylogenetic vs. epidemiologic). For the isolates of *M. tuberculosis* one standardization of the technique has been proposed by Supply *et al.* (2006). Therefore, this technique has been adopted for genotyping *M. tuberculosis* and *M. bovis* with encouraging results in terms of discrimination [223-225].

Forty one MIRU loci were identified in the chromosome of *M. tuberculosis* H37Rv, of which 12 showed polymorphisms in the number of tandem repeats, being designated variable number tandem repeats (VNTR) loci [218]. The most appropriate set of loci for the typing of *M. bovis* is not consensual and different laboratories may use different

selections. Also due to this reason, the spoligotyping remains the most widely used technique for the typing of *M. bovis* isolates. Nevertheless, the MIRU-VNTR analysis has proved useful for epidemiological studies, particularly when used in conjunction with other techniques, since it increases the power of discrimination between strains [226, 227].

1.4. Dissertation objectives and outline

The rapid detection and identification of *Mycobacterium tuberculosis* complex species is critical for the effective control of tuberculosis, with epidemiological importance to track outbreaks of zoonotic tuberculosis. The isolation and identification of these mycobacteria requires laboratory procedures typically complex and time consuming, resulting from the extremely tedious growth of these organisms in artificial culture media. The introduction of molecular biology techniques for the diagnosis of bovine tuberculosis, mostly based on the amplification and analysis of nucleic acids, has contributed to a more rapid detection and identification of the respective etiologic agent. However, the use of these techniques directly in biological samples often reveals a low sensitivity, also possibly associated with the presence of a small number of infective mycobacteria. The main global objective of this work was to develop improved molecular methods for the detection and discrimination of *Mycobacterium tuberculosis* complex species, particularly the species most often associated to animal tuberculosis. Ultimately, these methods will be adapted to marketable diagnostic molecular assays. This work was carried out in close cooperation between *Instituto Nacional de Investigação Agrária e Veterinária* (INIAV, I.P.) and *Unidade de Micobactérias do Instituto de Higiene e Medicina Tropical* (IHMT/UNL), under the framework of the novel OMS/OIE concept of "One World, One Health".

The following specific objectives were defined:

- To develop and validate an assay based on nested real-time PCR for the detection of *Mycobacterium bovis* and other MTC species directly in animal tissue samples;
- To implement a rapid diagnostic algorithm, based on TaqMan[®] chemistry, for the discrimination of the most veterinary-relevant *Mycobacterium tuberculosis* species;
- To develop an alternative molecular assay based in isothermal DNA amplification and lateral flow-based colorimetrically assessment of results to detect MTC members, able to be ultimately used in point-of-need and low-resource settings.

This dissertation is divided into five chapters. In the first chapter, a general theoretical introduction framing the subject under study is presented, with emphasis to the topics regarding the major characteristics of the *Mycobacterium tuberculosis* complex members, and bovine tuberculosis disease and its laboratorial diagnosis. In the second chapter, we describe the development of a semi-nested probe-based real-time PCR assay allowing an enhanced detection of tuberculous mycobacteria in animal tissues. In the third chapter, we describe a rapid identification algorithm for *Mycobacterium tuberculosis* complex species using specific dual-labelled hydrolysis probes in a multiplex format. In the fourth chapter, we describe the development of a duplex Loop-Mediated Isothermal DNA Amplification assay coupled with colorimetric lateral flow devices for the identification of *Mycobacterium tuberculosis* and *M. bovis*. Finally, in the fifth chapter, considerations are highlighted about the work presented and the main results that were achieved, and suggestions for future work.

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CHAPTER 2

**Enhanced detection of tuberculous mycobacteria in animal tissues
using a semi-nested probe-based real-time PCR**

Enhanced detection of tuberculous mycobacteria in animal tissues using a semi-nested probe-based real-time PCR

2.1. Summary

Bovine tuberculosis has been tackled for decades by costly eradication programs in most developed countries, involving the laboratorial testing of tissue samples from allegedly infected animals for detection of *Mycobacterium tuberculosis* complex (MTC) members, namely *Mycobacterium bovis*. Definitive diagnosis is usually achieved by bacteriological culture, which may take up to 6-12 weeks, during which the suspect animal carcass and herd are under sanitary arrest. In this work, a user-friendly DNA extraction protocol adapted for tissues was coupled with an IS6110-targeted semi-nested duplex real-time PCR assay to enhance the direct detection of MTC bacteria in animal specimens, reducing the time to achieve a diagnosis and, thus, potentially limiting the sanitary arrest period. The duplex use of a novel β -actin gene targeted probe, with complementary targets in most mammals, allowed the assessment of amplification inhibitors in the tissue samples. The assay was evaluated with a group of 128 fresh tissue specimens collected from bovines, wild boars, deer and foxes. *Mycobacterium bovis* was cultured from 57 of these samples. Overall, the full test performance corresponds to a diagnostic sensitivity and specificity of 98.2% (CI_{P95%} 89.4-99.9%) and 88.7% (CI_{P95%} 78.5-94.7%), respectively. An observed kappa coefficient was estimated in 0.859 (CI_{P95%} 0.771-0.948) for the overall agreement between the semi-nested PCR assay and the bacteriological culture. Considering only bovine samples (n = 69), the diagnostic sensitivity and specificity were estimated in 100% (CI_{P95%} 84.0-100%) and 97.7% (CI_{P95%} 86.2-99.9%), respectively. Eight negative culture samples exhibiting TB-like lesions were detected by the semi-nested real-time PCR, thus emphasizing the increased potential of this molecular approach to detect MTC-infected animal tissues. This novel IS6110-targeted assay allows the fast detection of tuberculous mycobacteria in animal specimens with very high sensitivity and specificity, being amenable and cost effective for use in the routine veterinary diagnostic laboratory with further automation possibilities.

2.2. Introduction

Tuberculosis (TB) is a leading cause of morbidity and mortality in the world, also affecting a wide range of animal species, particularly livestock, in both developed and developing countries. The disease is caused by tuberculous mycobacteria belonging to the *Mycobacterium tuberculosis* complex (MTC). This complex consists of several closely-related pathogenic species, namely *M. tuberculosis*, the main agent of human TB, and, amongst others, *M. bovis* and *M. caprae* that are primary agents of bovine and caprine TB, respectively [1]. These species are genetically very similar but may differ in host preference and epidemiological characteristics [2]. *Mycobacterium bovis* and *M. caprae* also represent a high potential for zoonotic transmission to humans [3, 4, 5], with evidence of possible person-to-person transmission [6]. However, the main routes of transmission are the contact with infected animals and ingestion of unpasteurized dairy products. These zoonotic MTC species may be responsible for up to 7.2% and 15% of human TB cases in industrialized and developing countries, respectively [7]. Rapid and reliable laboratory tests for the direct detection of tuberculous mycobacteria in biological samples are in high demand, in both human health and veterinary settings, and are crucial for an improved TB control.

In most developed countries, bovine tuberculosis has been tackled during the last decades by costly eradication programs, involving the culling of reactor animals and laboratorial testing of suspect samples for the definitive confirmation of the presence of MTC. Presently, the detection of MTC bacteria in animal tissues is mainly based in lengthy and cumbersome conventional methods, involving the examination of Ziehl-Neelsen stained smears, histopathology and culture in selective media, followed by biochemical or molecular identification of typical mycobacteria colonies. The microscopic identification of acid-fast mycobacteria is unspecific and highly insensitive, particularly in the presence of paucibacillary forms of TB. Culture remains the gold-standard method to confirm TB infection but requires several weeks to obtain positive results due to the extremely fastidious growth of tuberculous mycobacteria. In spite of the significant advances in the development of novel molecular diagnostic assays towards a faster and accurate detection of MTC in human samples, only a few assays were described for detecting these agents in animal tissues, particularly in fresh tissues from livestock [8-12]. Most of these molecular approaches are PCR-based and

target specific polymorphisms, insertion sequences, and the so-called regions of difference in the genome of MTC members [9; 10; 13-18]. Nevertheless, most of the amplification-based assays described for detecting MTC nucleic acids directly in fresh or formalin-fixed paraffin-embedded tissues only yield a moderate sensitivity, usually up to 75%, particularly when testing tissues without the characteristic lesions or detectable acid-fast bacilli [8-10; 15; 19]. This limitation is mostly related to the inefficiency of mycobacterial DNA extraction procedures from affected animal tissues, especially those exhibiting strong fibrosis, calcification, and with no histological evidence of acid-fast bacteria [8; 19]. The use of immunomagnetic separation approaches to concentrate mycobacteria cells from animal tissues prior to DNA extraction may enhance PCR sensitivities [20; 21]. Nevertheless, these approaches usually involve more experimental steps and expensive equipment and consumables not readily available in veterinary diagnostic settings.

In the present work we have developed a novel and simple *Taqman*-based semi-nested real-time PCR assay yielding extremely high sensitivity and specificity for the direct detection of tuberculous mycobacteria in fresh animal tissues, namely of bovine origin, capable of being introduced in the routine diagnostics of veterinary laboratories.

2.3. Materials and Methods

Bacterial strains

Reference strains and clinical isolates of MTC, non-MTC mycobacteria and non-mycobacterial species, maintained at the Portuguese reference laboratory for animal diseases (INIAV, IP), were used for optimization of PCR assays (Table 2.1). The identification of each isolate was based on standard methodologies [22]. MTC isolates were identified to the species level by PCR-restriction endonuclease analysis of the *gyrB* gene [23; 24; 25] and hybridization with species-specific probes [26].

Table 2.1. Bacterial reference strains and clinical isolates whose cultures were used in the present study for the evaluation of specificity of the amplification assays and respective results.

Species	Reference strains/Isolates	Presence of IS6110 ¹
<i>Mycobacterium tuberculosis</i>	ATCC 25177; LNIV 9605	+
<i>M. bovis</i>	AN5; ATCC 27291 (BCG); LNIV 13027; 5530/0/05; 11265; 7230/4; 14421/2; 24497/6; 8855; 5889; 10044; 14577; 13280/6; 13280/4; 34875; and 20564	+
<i>M. caprae</i>	LNIV 17320; 4958/0/05; 8403; 15244; and 20752	+
<i>M. avium</i> subsp. <i>avium</i>	ATCC 25291	-
<i>M. avium</i> subsp. <i>hominissuis</i>	LNIV 23063/4	-
<i>M. avium</i> subsp. <i>paratuberculosis</i>	LNIV 39888	-
<i>M. scrofulaceum</i>	LNIV 31389	-
<i>Acinetobacter baumannii</i>	LNIV 1628/12	-
<i>Arcanobacterium pyogenes</i>	VLA 1321	-
<i>Bacillus licheniformis</i>	VLA 1831	-
<i>Corynebacterium striatum</i>	LNIV 12352	-
<i>Enterobacter amnigenus</i>	LNIV 6050/II	-
<i>Klebsiella pneumonia</i>	VLA 1643	-
<i>Listeria monocytogenes</i>	VLA 1774	-
<i>Proteus mirabilis</i>	LNIV 2269/II	-
<i>Pseudomonas aeruginosa</i>	VLA 67	-
<i>Salmonella</i> Dublin	VLA 1272	-
<i>Staphylococcus aureus</i>	VLA 1032	-
<i>Streptococcus agalactiae</i>	VLA 33	-
<i>Yersinia enterocolitica</i>	VLA 1884	-

ATCC, American Type Culture Collection, USA; LNIV, Laboratório Nacional de Investigação Veterinária (currently INIAV, IP), Lisbon, Portugal; VLA, Veterinary Laboratory Agency, UK; ¹Amplification (+) or no amplification (-) of IS6110 element using the real-time PCR assay with P_IS6110 TaqMan probe and respective flanking primers F_IS6110 and R_IS6110.

Tissue samples

One hundred and twenty eight animal lymph nodes, liver, spleen or lung tissue samples (69 bovines, 35 wild boars, 15 deer and 9 foxes) were used in this work (Table 2.2). No animals were sacrificed for the purposes of this specific study. None of the authors were

responsible for the death of any animals and samples were originally collected for purposes other than research, namely: (i) bovine samples were collected from animals clinically suspected of having TB, e.g. by a positive reaction in either the single intradermal comparative tuberculin test or the gamma-interferon test, or TB-like lesions detected during routine abattoir inspection, and were submitted to routine control testing under the governmental Portuguese eradication scheme for bovine tuberculosis, approved in 1992 by the European Union (Council Decision 92/299/CE) and, since 2001, cofinanced by the European Union (in the framework of Council Directive 64/432, as amended) [27]; and (ii) TB suspect samples from wild boar, deer and fox, were sent to INIAV reference laboratories following gross pathological evaluation performed in the field by local veterinarians in hunting activities or predator control actions legally authorized by the "Instituto Nacional da Conservação da Natureza" (the Portuguese National Forest Authority), that emits permits for those hunting actions and for the respective hunters. Samples were submitted during the fourth trimester of 2011 to the pathology and bacteriology laboratories of INIAV and analysed using routine histological and culture-based methods, according to the OIE standard procedures [22] (Table 2.2). Tissues selected for bacteriological analysis were homogenized using a pestle and mortar, followed by decontamination with 4% sodium hydroxide. After neutralization with hydrogen chloride, the macerated suspensions were divided into equal parts. One part was maintained at -80 °C until further processing for molecular analysis and the other was centrifuged and the sediment inoculated into BACTEC 9000 liquid media and Lowenstein-Jensen with pyruvate and Stonebrink solid media. Inoculated media were incubated for a minimum of eight weeks at 37 °C. Heat-killed culture supernatants were kept at -20 °C until further testing. Species identification of presumptive mycobacteria isolates was based on the restriction endonuclease analysis with *RsaI* and *SacII* of the PCR-amplified *gyrB* gene, or using the commercial reverse hybridization assays INNO-LiPA Mycobacteria (Innogenetics, Belgium) or GenoType *Mycobacterium* (Hain diagnostics, Germany), following the manufacturer's instructions [23-25].

Table 2.2. Typology of tissue samples used in this study (n = 128) and respective results of the histological, bacteriological and semi-nested duplex real-time PCR analyses.

Sample typology	Number of tissue samples	Origin of tissues	Presence of lesions ¹	Bacteriological analysis ²	Nested real-time PCR ³
I	23	Bovine	+	<i>M. bovis</i>	23
	19	Wild boar	+	<i>M. bovis</i>	18
	12	Deer	+	<i>M. bovis</i>	12
II	3	Bovine	-	<i>M. bovis</i>	3
III	39	Bovine	-	-	0
	4	Wild boar	-	-	0
	3	Deer	-	-	0
IV	2	Bovine	-	Non-MTC	0
	3	Wild boar	-	Non-MTC	0
V	1	Bovine	+	-	0
	5	Wild boar	+	-	3
	6	Fox	+	-	0
VI	1	Bovine	+	Non-MTC	1
	4	Wild boar	+	Non-MTC	2
	3	Fox	+	Non-MTC	2

¹Presence of lesions compatible with tuberculosis; ²Detection of *M. bovis* or other non-MTC mycobacteria by culture of tissue samples; ³Number of samples for which the IS6110 element was amplified by the nested real-time PCR assay.

Spiked tissue samples

A lymph node tissue from a slaughtered bovine known to be free of TB, as confirmed by culture and histopathology, was used for testing as a spiked sample that was homogenized as described above. Eight aliquots containing 0.9 ml of tissue macerate were spiked with 0.1 ml of ten-fold dilutions of a suspension of *M. tuberculosis* H37Ra (ATCC 25177) cells. Estimated concentrations of mycobacteria in spiked macerate samples spanned from 10^7 to 10^0 cells/ml. An additional tube was spiked with 0.1 ml

PBS buffer and used as negative control. Spiked samples were stored at -20 °C until processing for DNA extraction.

DNA extraction from cultures

DNA extraction from cultures grown in liquid media was achieved by a combined bead beating and enzymatic extraction method described elsewhere [28]. Briefly, 0.5 ml bacterial culture were washed in PBS buffer and heat inactivated at 100 °C for 15 min. Tubes containing zirconium beads were used to resuspend the culture pellet in lysis buffer (SDS-EDTA-proteinase K) for mechanical disruption in the FastPrep 120 (Savant Instruments, Inc., Holbrook, NY) at 6.5 ms⁻¹ for 45 s, and then incubated overnight at 37 °C, followed by standard phenol–chloroform purification and ethanol precipitation of DNA. DNA concentration and purity were estimated by measuring the absorbance at 260 nm (A₂₆₀) and by A₂₆₀/A₂₈₀ and A₂₆₀/A₂₃₀ ratios, respectively, using a NanoDrop 1000 spectrophotometer (NanoDrop). Genomic DNA suspensions were stored at - 20 °C until further use.

DNA extraction from tissue macerates and spiked samples

Four hundred and fifty microliters of tissue suspensions were transferred to screw-cap microcentrifuge tubes and inactivated in a water bath at 100 °C for 5 minutes. Samples were centrifuged at 14000 rpm for 2 min, the supernatant rejected and 80 µl of PBS and an equivalent volume of 100 µl of zirconium beads were added to the tubes. After mechanical disruption in a FastPrep Bio 101 bead shaker (Savant Instruments Inc.) at 6.5 msec⁻¹ for 45 seconds, repeated three times, the suspensions were cooled on ice for 15 minutes. DNA extraction was carried out using the tissue protocol of the QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's instructions. The resulting genomic DNA suspensions were stored at - 20 °C until further use. Stock DNA suspensions were diluted ten times in distilled water before its use as template for PCR assays.

Design of TaqMan probes and flanking primers

Sequences of *IS6110* from MTC members and β -actin gene from a wide range of mammal species were retrieved from NCBI-GenBank. Comparative analysis of these two sets of sequences was achieved through sequence alignment using the CLUSTAL X v2.0 software [29]. Complementary regions for a β -actin gene-targeted mammals-universal TaqMan probe and flanking degenerated primers were found after visual inspection of the respective alignments (Table 2.3; Figure 2.1). The amplification of β -actin gene served as control to detect inhibition of the PCR reactions when using DNA extracted from tissues as template. The *IS6110*-targeted probe (P_IS6110) and respective flanking primers (F_IS6110 and R_IS6110) were retrieved from the study of Restrepo and colleagues [30] (Table 2.3; Figure 2.2). An additional *IS6110*-targeted forward primer (FN_IS6110) for use in a semi-nested PCR was designed (Table 2.3; Figure 2.2). Probes and primers specificities were tested in silico using the BLAST tools from NCBI-GenBank. All probes and primers targeting *IS6110* and β -actin gene were synthesized by MWG Biotech (Germany).

Table 2.3. Sequences of primers and probes used in this study.

Primer/ Probe	Sequence (5'-3')	Complementary target
F_Actin	GGC TCY ATY CTG GCC TC	β -actin gene of mammals
R_Actin	GCA YTT GCG GTG SAC RAT G	
P_Actin ¹	Cy5.5-TAC TCC TGC TTG CTG ATC CAC ATC-BHQ2	
F_IS6110	GGG TCG CTT CCA CGA TG	<i>IS6110</i> element of MTC species
FN_IS6110	CTC GTC CAG CGC CGC TTC GG	
R_IS6110	GGG TCC AGA TGG CTT GC	
P_IS6110 ²	FAM-CGC GTC GAG GAC CAT GGA GGT-BHQ1	

¹Probe labeled with Cy5.5 fluorophore and BHQ-2 quencher; ²Probe labeled with carboxyfluorescein (FAM) fluorophore and BHQ-1 quencher

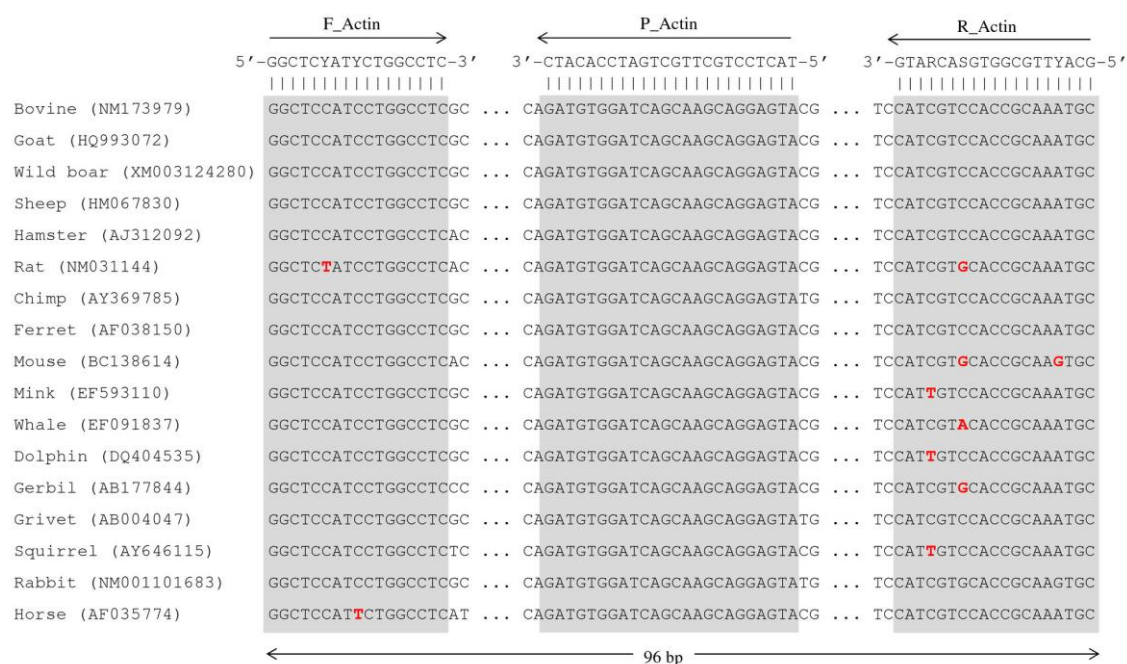


Figure 2.1. Complementary targets of the mammals β -actin gene targeted probe and flanking primers. Partial alignment of β -actin gene sequences of several mammal species showing the complementary targets of the P_Actin *TaqMan* probe and respective flanking degenerated primers (F_Actin and R_Actin) (gray boxes). The GenBank access numbers from which the partial sequences were retrieved are indicated for each species inside parenthesis. Mismatches in relation to consensus sequence are highlighted in red.

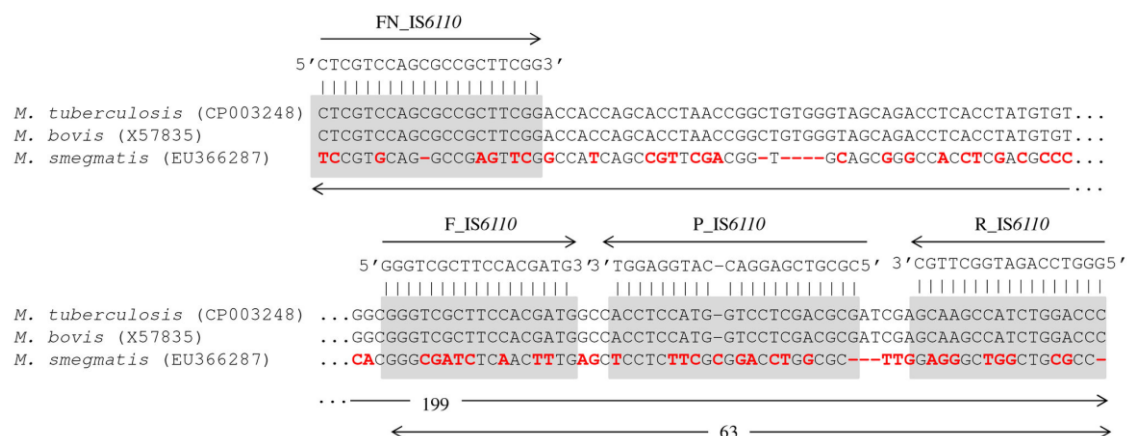


Figure 2.2. Complementary targets of the MTC-specific IS6110-targeted probe and flanking primers. Partial alignment of the IS6110 sequence of *Mycobacterium tuberculosis* complex members with other IS6110-like sequence recently found in *M. smegmatis* (GenBank access numbers are indicated inside parenthesis). The complementary targets of the P_IS6110 *TaqMan* probe and flanking primers (FN_IS6110, F_IS6110 and R_IS6110) are indicated (gray boxes). Mismatches in sequences are highlighted in red.

Amplification assays

The semi-nested real-time PCR amplification assay using DNA extracted from tissue samples as template consisted of two steps: (i) a first standard PCR using primers FN_IS6110 and R_IS6110; and (ii) a second duplex real-time PCR using the previous amplification product as template and a mixture of IS6110 and β -actin gene targeted TaqMan probes (P_IS6110 and P_Actin, respectively) and the corresponding flanking primers (F_IS6110/R_IS6110 and F_Actin/R_Actin, respectively) (Table 2.3). IS6110-targeted amplification reactions were previously optimized using DNA extracted from pure cultures as template. The first standard PCR step amplifies an MTC-specific 199 bp fragment of the IS6110 (Figure 2.2). PCR reactions were carried out in a final volume of 25 μ l containing 400 μ M of deoxynucleotide triphosphates (Promega), 1 U of Taq DNA polymerase (Promega), 3.5 mM of MgCl₂ (Promega), 0.8 μ M of each primer (FN_IS6110 and R_IS6110), DNase free water (GIBCO) and 5 μ l of extracted DNA template. Amplification was performed in a C1000 thermocycler (Bio-Rad) using the following program: initial denaturation step at 95 °C for 10 min, 45 cycles of 30 sec at 95 °C, 30 sec at 65 °C, 30 sec at 72 °C, and a final extension step of 10 min at 72 °C. The amplified products were stored at 4 °C until electrophoresis analyses in a 2% agarose gel or directly used for the second duplex real-time PCR step. Real-time PCR reactions were carried out in a total volume of 20 μ l containing 1 \times SSO Fast Super Mix (Bio-Rad), 0.4 μ M of each primer, 0.15 μ M of TaqMan probe, DNase free water (GIBCO) and 5 μ l of the previous PCR products. The concentrations of primers and probes were maintained when used in the duplex format. The thermal cycling conditions were as follows: 1 cycle at 95 °C for 2 min, followed by 45 cycles at 95 °C for 5 s and 60 °C for 10 s. All samples that probed positive for IS6110 were retested for confirmation. Thermal cycling, fluorescent data collection, and data analysis were performed in a CFX96 (Bio-Rad) detection system real-time PCR instrument, according to the manufacturer's instructions.

Analytical specificity and sensitivity

To investigate whether the IS6110-targeted real-time PCR assay specifically amplifies DNA from MTC members, MTC and non-MTC mycobacterial isolates were tested, as

well as other clinically relevant bacteria (Table 2.1). To estimate the detection threshold of the assay (analytical sensitivity), a standard curve was constructed using 10-fold serial dilutions of DNA extracted from *M. tuberculosis* H37Ra (ATCC 25177) as template. Each template was run in triplicate. The end-point corresponded to the dilution at which the assay could not detect the target in at least one of the replicates.

Detection limit of the IS6110-targeted semi-nested real time PCR assay

The detection limit of the semi-nested duplex real time PCR assay was assessed using the serially spiked tissue macerate samples. Each template was tested in triplicate.

Diagnostic specificity and sensitivity

The tissue samples were stratified in six typologies (Table 2.2): I - lesions compatible with tuberculosis are present and *M. bovis* was cultured from the samples (n = 54); II- lesions are not present but *M. bovis* was cultured from samples (n = 3); III- absence of any lesions and mycobacteria were not cultured from samples (n = 46); IV- absence of lesions but non-MTC mycobacteria were cultured from samples (n = 5); V- lesions compatible with tuberculosis are present but mycobacteria were not cultured (n = 12); and VI - lesions are present and non-MTC mycobacteria were cultured from samples (n = 8). The culture of *M. bovis* from tissue samples was used as gold-standard reference method for the computation of the diagnostic sensitivity and specificity of the semi-nested real-time PCR assay. Overall diagnostic sensitivity, specificity and positive (PPV) and negative (NPV) predictive values were computed using all culture positive (types I + II; n = 57) and negative (types III, IV, V and VI; n = 71) tissue samples (Table 2.2). Additionally, these parameters were also computed using only the bovine culture positive (n = 26) and negative (n = 43) samples (Table 2.2). Although sample types V and VI were also culture negative for *M. bovis*, lesions compatible with the disease were found. For the computation of the kappa coefficient, for measuring the agreement between the gold-standard method of bacteriological culture and the IS6110-targeted semi-nested PCR assay, all tissue samples were used (types I - VI, n = 128). Sensitivity, specificity, PPV, NPV and kappa coefficient, with confidence intervals,

were computed using the clinical research calculators of the online VassarStats software (<http://vassarstats.net>).

2.4. Results

Design of probes and primers

A novel set of *β-actin* gene-targeted *TaqMan* probe and respective flanking primers was designed (Table 2.3, Figure 2.1). *In silico* analysis using the BLAST suite of NCBI-GenBank confirmed that the complementary regions of this probe, and primers, are widespread amongst most mammal species, including livestock animals. *In silico* analysis also confirmed that the complementary regions of the *IS6110*-targeted probes and primers were only present in *IS6110* sequences of MTC members (Table 2.3, Figure 2.2).

Analytical specificity and sensitivity

The real-time PCR assay with the *IS6110*-targeted probe (P_*IS6110*) and flanking primers (F_*IS6110* and R_*IS6110*) yielded amplification products only when using DNA extracted from MTC members as template (Table 2.1, Figure 2.3). No non-specific results were obtained with members of the *Mycobacterium avium* complex or with strains belonging to other diverse bacterial species. The minimum detection threshold (analytical sensitivity) of this assay was estimated by the construction of a reference curve with serially-diluted suspensions of DNA from *M. tuberculosis* H37Ra. The analytical sensitivity was estimated in 0.3 fg/μL of *M. tuberculosis* genomic DNA. A linear relationship between the logarithm of the starting concentration of DNA and the amplification Ct values was obtained, with a -3.222 slope, a Ct = 35 interception in the minimum threshold (0.3 fg/μL) and an $R^2 = 0.999$.

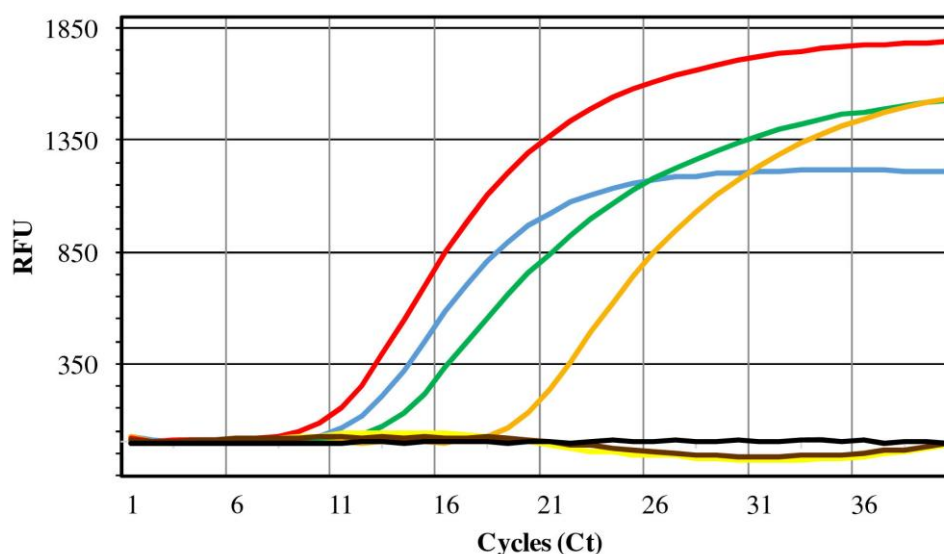


Figure 2.3. IS6110-targeted real-time PCR amplification curves obtained in specificity tests. Illustration of the MTC-specific amplification curves obtained in FAM channel during evaluation of the analytical specificity of the IS6110-targeted real-time PCR step. All MTC cultures yielded amplification curves: *M. tuberculosis* ATCC 25177 (blue line), *M. bovis* LNIV 13027 (green line), *M. bovis* ATCC 27291 (orange line) and *M. caprae* LNIV 17320 (red line). Other bacteria yielded negative results of amplification: *M. avium* subsp. *avium* ATCC 25291 (yellow line) and *M. avium* subsp. *paratuberculosis* LNIV 39888 (brown line). No amplification was detected in non-template negative control (black line). RFU - Relative Fluorescence Units.

Spiked samples

Tissue samples spiked with serially-diluted suspensions of *M. tuberculosis* cells were used for assessing the detection limit of the β -actin and IS6110-targeted semi-nested duplex real-time PCR assay. The detection threshold was estimated to be one mycobacteria per ml of tissue macerate (Figure 2.4). The IS6110 amplification curves usually harboured a very low Ct (< 3), meaning that the first standard PCR step yielded abundant amplification products easily detected by the following real time PCR step. The co-amplification of the bovine β -actin gene showed that no apparent inhibition of the real-time PCR step occurred due to the presence of inhibitor components of tissues or excess of bovine DNA, a significant result for the type of specimens used (animal tissues), confirming the efficiency of the extraction and purification procedures used in this work (Figure 2.4).

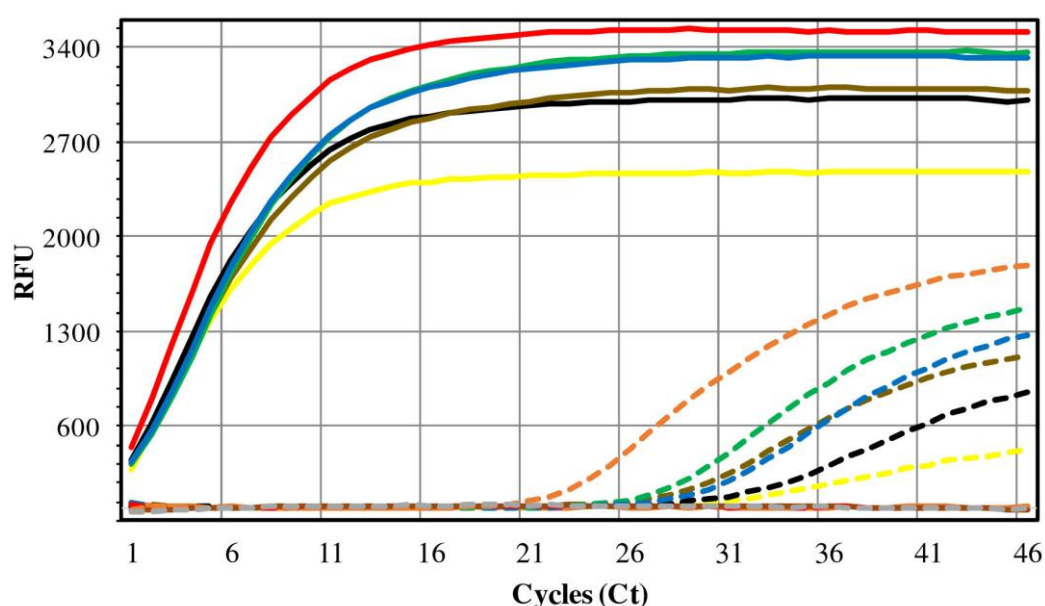


Figure 2.4. Testing of spiked samples with the semi-nested duplex real-time PCR assay. The analytical sensitivity of the assay was estimated through the use of solutions of DNA extracted from tissue samples spiked with serially diluted cellular suspensions of *M. tuberculosis* ATCC 25177 (ranging from 10^7 to 10^0 cells/ml of tissue homogenate). The figure illustrates the IS6110 (solid lines) and mammal β -actin gene (dashed lines) targeted amplification curves obtained in FAM and Cy5.5 channels, respectively, for the several dilutions: 10^7 (yellow line), 10^5 (black line), 10^3 (brown line), 10^1 (green line) and 10^0 (blue line) cells. Positive control: *M. tuberculosis* ATCC 25177 culture (red line); Negative controls: unspiked tissue homogenate (orange line) and non-template control (grey line). RFU - Relative Fluorescence Units.

Diagnostic specificity and sensitivity

The results of the semi-nested duplex real-time PCR assays using DNA extracted from tissue samples are summarized in Table 2.2. All but one tissue sample from which *M. bovis* was cultured (types I and II) yielded positive amplification results, corresponding to a diagnostic sensitivity of 98.2% (CI_{95%} 89.4-99.9%). Eight DNA-positive tissues were obtained among the 71 *M. bovis* culture negative samples (types III, IV, V and VI), yielding a diagnostic specificity of 88.7% (CI_{95%} 78.5-94.7%). Negative controls run with batches of samples did not identify any cross-contaminating DNA. The positive and negative predictive values were estimated in 87.5% (CI_{95%} 76.3-94.1%) and 98.4% (CI_{95%} 90.1-99.9%), respectively, for a prevalence of 44.5% *M. bovis* culture positive samples among all samples analysed. Considering only the bovine samples, the diagnostic sensitivity and specificity of the amplification assay were

estimated in 100% (CI_{P95%} 84.0-100%) and 97.7% (CI_{P95%} 86.2-99.9%), respectively, while PPV and NPV were estimated in 96.3% (CI_{P95%} 79.1-99.8%) and 100% (CI_{P95%} 89.6-100%), respectively, for a prevalence of 37.7% *M. bovis* culture positive samples.

No DNA-positive samples were obtained from samples without TB-compatible lesions and *M. bovis* isolation (types III and IV) (Table 2). However, eight DNA-positive samples were detected among tissues that were *M. bovis* culture-negative but that harboured TB-compatible lesions (sample types V and VI) (Table 2.2).

An observed kappa coefficient was estimated in 0.859 (CI_{P95%} 0.771-0.948) for the overall agreement between the results obtained by the direct application of the IS6110-targeted semi-nested PCR assay to fresh tissue samples and the results obtained from the gold-standard bacteriological culture. The direct detection procedure allowed the detection of MTC infected samples in less than 6 hours while the conventional culture takes about 6 to 12 weeks.

2.5. Discussion

The availability of TB confirmatory tests allowing a fast and conclusive detection of tuberculous mycobacteria in suspect animal tissues would be a great advantage in improving the efficiency of TB eradication programs and in decreasing the associated economic burden. Improvements in specificity, sensitivity and detection limit of diagnostic assays are usually introduced by molecular approaches. Nevertheless, only few diagnostic PCR-based assays were described for detecting MTC members directly in animal specimens, usually yielding limited sensitivities when compared to the reference bacteriological culture [8-12; 15; 19]. These moderate sensitivities may well be linked to the increased complexity for disrupting and recovering genomic DNA from the tough mycobacterial cells and to the paucibacillary nature of many animal tissue lesions used for nucleic acids extraction [10]. The presence of amplification inhibitors in crude tissue extracts, namely of large amounts of co-extracted eukaryotic DNA, may represent an additional problem. An option that has been explored to enhance the sensitivity of PCR techniques involves the implementation of more effective DNA extraction and purification methods, for example by using sequence capture or immunomagnetic separation approaches [8; 9; 12; 21]. Nevertheless, these approaches

usually involve more experimental steps and eventually expensive and not readily available equipment and consumables.

In this work, an alternative approach was evaluated for enhancing MTC detection sensitivities directly from fresh animal tissues. An adapted and optimized user-friendly DNA extraction protocol, mainly based in the use of simple commercially obtainable extraction kits, was coupled with an *IS6110*-targeted semi-nested real-time PCR assay that allows the direct detection of MTC members in animal tissue specimens with very high sensitivity, specificity and positive and negative predictive values, namely in bovine specimens. These two last parameters are dependent on the prevalence of the tested condition in the population under study, as well as on the sensitivity and specificity of the testing assays. The prevalence of MTC (mostly *M. bovis*) infected samples among all TB-suspect samples submitted to the reference laboratory during the 2002-2010 period ($n = 6364$) was approximately 40% (reviewed by Cunha and colleagues [27]), which compares well with the prevalence values found in this work and used for the computation of PPV and NPV. Therefore, the PPV and NPV parameters computed may be considered good indicators of the performance of our *IS6110*-targeted assay, when the same is to be used for assessing the presence of MTC in specimens collected from animals clinically suspected of having tuberculosis, namely cattle, submitted for confirmatory culture analysis in a major reference laboratory.

Eight DNA-positive amplification results were obtained from tissue samples from which *M. bovis* could not be cultured (Table 2.2). However, lesions compatible with TB were observed in these tissues during histological analysis (sample types V and VI) (Table 2.2). The culture of tissue samples for the isolation of *M. bovis* and of other MTC members, followed by molecular or biochemical identification procedures, is usually the gold-standard method to validate alternative diagnostic assays. However, it is known that bacteriological culture is slow and laborious and can yield ambiguous or false-negative results, e.g. due to the presence of non-viable mycobacteria, raising concerns about its effectiveness as comparison reference method [19; 31]. The results of culture assays may be affected by several factors, such as the harsh processing and decontamination procedures of samples, which can also have a harmful effect on *M. bovis* viability, as well as the growth media and incubation conditions used and the constrained distribution of mycobacteria in tissues [32]. Therefore, although TB-like

lesions identified by histopathology can be induced by other bacteria or mycobacteria, the positive amplification results suggest that MTC members were most probably associated with the observed lesions and that the PCR test is more sensitive than bacteriological culture for detecting these pathogens in animal tissues. Non-MTC mycobacteria such as *M. avium* and *M. scrofulaceum* were cultured from five of those eight DNA-positive tissue samples (Table 2.2). Eventually, any *M. bovis* isolates present in the sample may have been overgrown by these non-MTC mycobacteria [19]. Regardless of some discrepancies, the kappa measure of agreement between bacteriological culture and the semi-nested PCR was estimated in 0.859. Although the criteria for judging kappa statistic are not completely objective nor universally accepted, this value may allow to infer an "almost perfect" agreement between both MTC-detection methods [33].

The enhancement of the MTC detection rates using the semi-nested amplification assay need to be balanced against the associated increased risk of cross contamination of samples. However, the occurrence of false-positive results was not an issue in this work, while working in a veterinary diagnosis laboratory harboring standard conditions for molecular analysis, which include working in separate clean rooms and the use of positive and negative controls.

In a preliminary survey the testing of TB positive and negative tissue samples employed a standard real-time PCR assay (corresponding to the second step of the semi-nested approach). The comparison of this assay with the reference of bacteriological culture showed a diagnostic sensitivity of only 40% (data not shown). The inclusion of the first step of conventional IS6110-targeted PCR amplification, in a semi-nested design, allowed to increase the sensitivity of the assay to near 100%. Previous studies found no significant improvements in the detection of MTC members in animal tissue samples using nested PCR assays, including with real-time PCR formats [10; 14; 15]. Nevertheless, the performance of PCR detection systems is highly dependent on the efficiency of the primers and probes, even when using the same genomic targets such as the IS6110 element [34]. The MTC-specific IS6110-targeted primers and probe used in this work showed to be highly efficient for detecting tuberculous mycobacteria in animal tissue samples, although the isolates of the most relevant species, *M. bovis*, usually contain only one copy of this insertion sequence. It has recently been found that

IS6110-like elements may be present in other non-MTC mycobacteria such as *M. smegmatis* [35]. However, the probe and respective flanking primers used show no relevant complementary regions with these IS6110-like nucleotide sequences (Figure 2.2).

The protocol for direct detection of MTC from fresh animal tissues, using an optimized DNA extraction and purification procedure coupled with a semi-nested real-time PCR assay described in this work, revealed to be highly accurate and much faster than bacteriological culture, reducing the time for confirmatory TB diagnosis from several weeks to few hours, thus also potentially decreasing the sanitary arrest period of the suspect animal carcass and herd. The assay is amenable to future automation possibilities regarding both the DNA extraction and amplification steps. It may also allow the detection of MTC members when these pathogens become nonviable and non-cultivable or are overgrown by other less fastidious bacteria or mycobacteria also present in tissue samples. The use of a novel β -actin gene targeted probe, and respective flanking primers, with complementary targets in most mammal species, allowed to assess the presence of amplification inhibitors in the DNA extracts. Although the test is not able to distinguish between different members of the MTC, particularly *M. bovis*, the identification of any tuberculous mycobacteria infection in domestic or wildlife animals would warrant a public health awareness.

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CHAPTER 3

Rapid identification of veterinary-relevant *Mycobacterium tuberculosis* complex species using 16S rDNA, IS6110 and Regions of Difference-targeted dual-labelled hydrolysis probes

Rapid identification of veterinary-relevant *Mycobacterium tuberculosis* complex species using 16S rDNA, IS6110 and Regions of Difference-targeted dual-labelled hydrolysis probes

3.1. Summary

Members of the *Mycobacterium tuberculosis* complex (MTC) are causative agents of tuberculosis (TB) in both humans and animals. MTC species are genetically very similar but may differ in their epidemiology, namely geographic distribution and host preferences, virulence traits and antimicrobial susceptibility patterns. However, the conventional laboratory diagnosis does not routinely differentiate between the species of the MTC. In this work we describe a rapid and robust two-step five-target probe-based real-time PCR identification algorithm, based on genomic deletion analysis, to identify the MTC species most commonly associated with TB in livestock and other animals. The first step allows the confirmation of the cultures as MTC members, by targeting their IS6110 element, or as a mycobacterial species, if only a 16S rDNA product is detected in the duplex amplification reaction. If a MTC member is identified, the second amplification step allows to assess the presence or absence of the RD1, RD4 and RD9 genomic regions. The correspondent pattern allows to infer the species of the isolate as *M. tuberculosis* (if all RDs are present), *M. caprae* (if only RD1 and RD4 are present) and *M. bovis* (if only RD1 is present). The identification algorithm developed presented an almost perfect agreement with the results of the routine bacteriological analysis, with a kappa coefficient of 0.970 (CI_{p95%} 0.929-1.000). The assay is able to be adaptable to automation and implementation in the routine diagnostics framework of veterinary diagnostics laboratories, with a particular focus for reference laboratories.

3.2. Introduction

Members of the *Mycobacterium tuberculosis* complex (MTC) are causative agents of tuberculosis (TB) in both humans and animals. This complex encompasses several closely-related pathogenic species, notably *M. tuberculosis* and *M. africanum*, main agents of human TB, *M. bovis*, primarily linked to bovine TB but also often isolated from a wide range of other domestic and wild animals, and *M. caprae*, mostly

associated to caprine TB [1-3]. Other less frequently found MTC species associated to TB disease in animals are *M. pinnipedii*, *M. microti*, *M. mungi* and *M. orygis* [4-10]. MTC species, particularly *M. bovis* and *M. caprae*, represents a high potential for zoonotic transmission to humans [3, 11, 12], with evidence for the possibility of person-to-person transmission [13, 14]. However, the main routes of transmission are the contact with infected animals and ingestion of unpasteurized dairy products [15].

MTC species are genetically very similar but may differ in their epidemiology, namely geographic distribution and host preferences, virulence traits and antimicrobial susceptibility patterns. The discrimination between these species is therefore important for the accurate diagnosis and epidemiological assessment of mycobacterial disease in both humans and animals, public health surveillance and, if required, appropriate therapy management. Bacteriological culture, followed by biochemical and/or molecular identification, remains the gold-standard method to confirm MTC infection, a time consuming process due to the extremely fastidious growth of these mycobacteria, that usually do not differentiate species inside the MTC. In Portugal the National Eradication Program for bovine tuberculosis is based on systematic tuberculin testing of cattle, slaughter of positive animals, post-mortem inspection and histopathological and bacteriological diagnosis at the National Reference Laboratory (INIAV IP) [16-19]. Other domestic and wild animals such as deer and wild boar have been also frequently tested for TB in this reference laboratory [18, 19, 20]. However, the conventional laboratory diagnosis does not routinely differentiate between the species of the MTC. In most circumstances the culture-based phenotypic and biochemical assays able to discriminate between at least some of the MTC species are slow, cumbersome and time consuming, partially due to the fastidious growth of these mycobacteria, may yield ambiguous results and may be limited by a degree of technical complexity that rule out its routine implementation in most veterinary laboratories [21]. These assays may include the assessment of oxygen requirements, niacin accumulation, nitrate reductase activity, resistance to pyrazinamide, and use of glycerol and pyruvate by the MTC strains [22, 23].

MTC members harbour identical 16S rDNA sequences, which is the most commonly used genomic target in bacteriology for species identification [24]. Nevertheless, in the last two decades, the accumulating knowledge of the nucleotide sequences of several

genes, and of the whole genomes, of MTC members have allowed the development of novel molecular assays able to discriminate between these species. Approaches such as the analysis of single nucleotide polymorphisms (SNPs) of the *gyrB* gene, and of other genes, or the assessment of the structure of variable repetitive regions (e.g. direct repeats) or of large sequence polymorphisms (LSPs) in the genome of MTC members have been described that effectively discriminate between these species [21, 25-30], if properly framed in an adequate laboratory diagnosis algorithm. Most of these molecular approaches are developed 'in house', are PCR-based and involve post-amplification processing, such as gel electrophoresis analysis, multistep reverse hybridizations or DNA sequencing. As far as we know, there is only one commercially available, reverse-hybridization based, molecular test for the differentiation of MTC members (the Genotype MTBC assay, Hain Lifesciences GmbH, Germany).

Comparative genomic analysis evidenced that modern MTC species probably evolved from a common ancestor through the accumulation of sequential and irreversible genomic deletions, named Regions of Difference (RDs), which has been important in generating genetic diversity within these closely related mycobacteria [1, 2, 31-34]. The pattern of the presence or absence of these RDs in the genome of MTC members provides a molecular signature that can clearly discriminate among these mycobacteria [1, 2, 8, 10]. Several RDs-targeted amplification-based assays have been previously proposed for MTC species discrimination, usually involving standard PCR reactions and analysis of products by gel electrophoresis [15, 35, 36], real-time PCR using intercalating fluorescent dyes and melting curve analysis [28, 37] and, to a lesser extent, dual labelled hydrolysis probes [30, 38, 39]. All these assays were predominantly developed for use in human TB laboratorial settings.

In this work we report the design, development and testing of a rapid and robust *TaqMan*[®]-based real-time PCR identification algorithm, based on genomic deletion analysis, to distinguish between the members of the MTC most commonly associated with TB in livestock and other animals, able to be used in the routine diagnostics framework of veterinary diagnostics laboratories.

3.3. Materials and Methods

Bacterial strains

Reference strains and clinical isolates of MTC, non-MTC mycobacteria and non-mycobacterial species, maintained at the Portuguese reference laboratory for animal diseases (*Instituto Nacional de Investigação Agrária e Veterinária*, INIAV, IP) (animal isolates) and at the *Instituto de Higiene e Medicina Tropical* (IHMT/UNL) (human isolates), were used for optimization of PCR assays (Table 3.1). MTC isolates were previously identified to the species level by PCR-restriction endonuclease analysis of the *gyrB* gene [16, 17, 20] (at INIAV, I.P.) or by using the Genotype MTBC assay (Hain Lifesciences GmbH, Germany) (at IHMT/UNL).

Table 3.1. Reference and clinical strains used in the present study and respective results for the duplex (16S rDNA and *IS6110*) and triplex (Rv3875-RD1, Rv1510-RD4 and Rv2073c-RD9) genomic targets amplification.

Species	Strains	16S rDNA	<i>IS6110</i>	RD1	RD4	RD9
<i>M. tuberculosis</i>	ATCC 25177; LNIV 9605; IHMT 1; 2; 3; 4; 5; 6; 7; 8; 9; 10; 11; 12; 13; 14; and 15	+	+	+	+	+
<i>M. africanum</i>	IHMT 39; 46; 47; and 49	+	+	+	+	-
<i>M. caprae</i>	LNIV 17320; 4958/0/05; 8403; 15244; and 20752	+	+	+	+	-
<i>M. bovis</i>	AN5; LNIV 13027; 5530/0/05; 11265; 7230/4; 14421/2; 24497/6; 8855; 5889; 10044; 14577; 13280/6; 13280/4; 34875; and 20564	+	+	+	-	-
<i>M. bovis</i> BCG	ATCC 27291 (BCG); IHMT 36; 37; 48 and 50	+	+	-	-	-
<i>M. avium</i> subsp. <i>avium</i>	ATCC 25291	+	-	-	-	-

Table 3.1. (cont.)

Species	Strains	16S rDNA	IS6110	RD1	RD4	RD9
<i>M. avium</i> subsp. <i>hominissuis</i>	LNIV 23063/4; IHMT 31/06; 229/06; 193/08; 189/05; 8/05; 291/05; 386/08; 92/06; 78/06; 327/07; 73/11; 436/05; and 68/06	+	-	-	-	-
<i>M. avium</i> subsp. <i>paratuberculosis</i>	LNIV 39888	+	-	-	-	-
<i>M. scrofulaceum</i>	LNIV 31389	+	-	-	-	-
<i>Acinetobacter</i> <i>baumannii</i>	LNIV 1628/12	-	-	-	-	-
<i>Arcanobacterium</i> <i>pyogenes</i>	VLA 1321	-	-	-	-	-
<i>Bacillus</i> <i>licheniformis</i>	VLA 1831	-	-	-	-	-
<i>Corynebacterium</i> <i>striatum</i>	LNIV 12352	-	-	-	-	-
<i>Enterobacter</i> <i>amnigenus</i>	LNIV 6050/II	-	-	-	-	-
<i>Klebsiella</i> <i>pneumonia</i>	VLA 1643	-	-	-	-	-
<i>Listeria</i> <i>monocytogenes</i>	VLA 1774	-	-	-	-	-
<i>Proteus mirabilis</i>	LNIV 2269/II	-	-	-	-	-
<i>Pseudomonas</i> <i>aeruginosa</i>	VLA 67	-	-	-	-	-
<i>Salmonella</i> Dublin	VLA 1272	-	-	-	-	-
<i>Staphylococcus</i> <i>aureus</i>	VLA 1032	-	-	-	-	-
<i>Streptococcus</i> <i>agalactiae</i>	VLA 33	-	-	-	-	-
<i>Yersinia</i> <i>enterocolitica</i>	VLA 1884	-	-	-	-	-

ATCC, American Type Culture Collection, USA; LNIV, Laboratório Nacional de Investigação Veterinária (currently INIAV, IP), Lisbon, Portugal; IHMT, Instituto de Higiene e Medicina Tropical, Lisbon, Portugal; VLA, Veterinary Laboratory Agency, UK; Amplification (+) or no amplification (-).

Genomic DNA extraction from purified reference and clinical bacterial strains

DNA extraction from cultures grown in liquid media was achieved by a combined bead beating and enzymatic extraction method described elsewhere [40]. Briefly, 0.5 ml bacterial culture were washed in PBS buffer and heat inactivated at 100 °C for 15 min. Tubes containing zirconium beads were used to resuspend the culture pellet in lysis buffer (0.4 M NaCl, 40 mM Tris-HCl, pH 8, 2 mM EDTA, 0.6% SDS, 0.034 mg/ml proteinase K) for mechanical disruption in the FastPrep FP120 Bio101 (Savant Instruments, Inc., Holbrook, NY) at 6.5 ms⁻¹ for 45 s, and then incubated overnight at 37 °C, followed by standard phenol–chloroform purification and ethanol precipitation of DNA. DNA concentration and purity were estimated by measuring the absorbance at 260 nm (A₂₆₀) and by A₂₆₀/A₂₈₀ and A₂₆₀/A₂₃₀ ratios, respectively, using a NanoDrop 1000 spectrophotometer (NanoDrop). Genomic DNA suspensions were stored at - 20 °C until further use.

Design of *TaqMan* probes and respective flanking primers

Sequences of the 16S rDNA gene and of the Regions of Difference 1 (RD1, including the Rv3875-*esat6* locus), 4 (RD4, including the Rv1510 locus) and 9 (RD9, including the Rv2073c locus) from MTC members were retrieved from NCBI-GenBank. Comparative analysis of these four sets of sequences was achieved through sequence alignment using the CLUSTAL X v2.0 software [41]. Probes and primers were designed and specificities were assessed *in silico* using the BLAST tools from NCBI-GenBank. All probes and primers were synthesized by MWG Biotech (Germany).

Mycobacterial 16S rDNA-targeted primers were obtained from the study of Richardson and colleagues [42] (forward primer) and Kirschner and colleagues [43] (reverse primer), and an additional *TaqMan* probe complementary to the amplified product was designed (Table 3.2). Additionally, an IS6110-targeted probe and respective flanking primers were retrieved from the study of Restrepo and colleagues [44] (Table 2).

Complementary regions for Rv3875-*esat6* (RD1), Rv1510 (RD4) and Rv2073c (RD9) loci targeted *TaqMan* probes and respective flanking primers were found after analysis of the respective nucleotide sequences (Table 2).

Table 3.2. Sequences of primers and probes used in this study.

Primer/Probe	Sequence (5'-3')	Product length (bp) ¹	Complementary target
F_16SrDNA	CCG CAA GGC TAA AAC TCA AA	149	Mycobacterial 16S rDNA
R_16SrDNA	TGC ACA CAG GCC ACA AGG GA		
P_16SrDNA ²	TET-TCG ATG CAA CGC GAA GAA CCT TAC- BHQ1		
F_IS6110	GGG TCG CTT CCA CGA TG	63	IS6110 element of MTC
R_IS6110	GGG TCC AGA TGG CTT GC		
P_IS6110 ³	FAM-CGC GTC GAG GAC CAT GGA GGT-BHQ1		
F- <i>esat6</i>	AGG CGT ACC AGG GTG TC	110	RD1 (Rv3875 locus, <i>esat6</i> gene)
R- <i>esat6</i>	CGA AGC CAT TGC CTG ACC		
P- <i>esat6</i> ⁴	Cy5.5-ACA ACG CGC TGC AGA ACC TGG-BHQ2		
F-Rv1510	CCT GCA AGA AAC GAC CCG	105	RD4 (Rv1510 locus)
R-Rv1510	GCG ACG GTG CCA ATC ATC		
P-Rv1510 ⁵	TET-CCA TCG TAC CCA TCC GCT GCG-BHQ1		
F-Rv2073c	AGT CGG TGT GCA CGA TGG	117	RD9 (Rv2073c locus)
R-Rv2073c	CGC TCG TTG CCG AGC AC		
P-Rv2073c ⁶	Texas Red-CTG GTC GCC GAG TAT CCC GAA G- BHQ2		

¹As retrieved from the whole genome sequence of *M. tuberculosis* strain H37Rv (GenBank Accession Number AL123456); ²Probe labeled with TET (tetrachlorofluorescein) fluorophore and BHQ1 quencher; ³Probe labeled with FAM (carboxyfluorescein) fluorophore and BHQ1 quencher; ⁴Probe labeled with Cy5.5 fluorophore and BHQ2 quencher; ⁵Probe labeled with TET fluorophore and BHQ1 quencher; ⁶Probe labeled with Texas red fluorophore and BHQ2 quencher.

The complementary regions of the P-*esat6*, F-*esat6* and R-*esat6* probe and flanking primers, respectively, are located in the 6 kDa early secretory antigenic target gene (*esat6*, Rv3875 locus) (as in the sequence with GenBank accession number AL123456, region 4352609 - 4352896, of the whole genome of *M. tuberculosis* strain H37Rv), included in the RD1 genomic region, which is present in most MTC members with the exception of the vaccine *M. bovis* BCG strains (Figure 3.1).

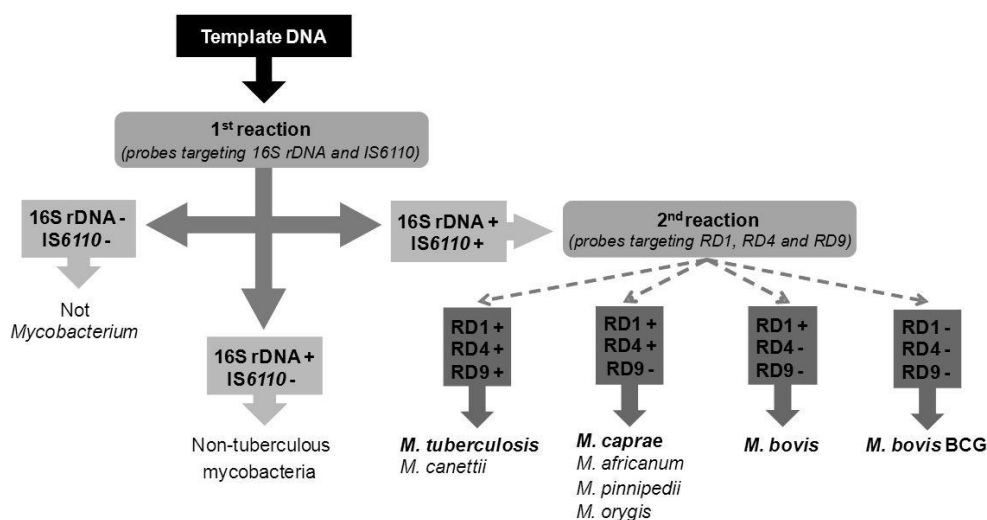


Figure 3.1. MTC testing algorithm. Schematics of the two-step five-target *TaqMan*[®]-based real-time PCR identification algorithm, based on genomic deletion analysis, to identify the MTC species most commonly associated with TB in livestock and other animals. The target locus of each RD is as follows: Rv3875-*esat6* for RD1, Rv1510 locus for RD4 and Rv2073c locus for RD9. Due to specific deletions spanning at least part of the region RD1, including the Rv3875-*esat6* locus, the profile expected in the second triplex reaction for potential isolates of *M. microti* and *M. mungi* would be: RD1 (-), RD4 (+) and RD9 (-).

Other MTC members also present specific RD1 deletions, namely *M. mungi* (RD1^{mun}) [9] and *M. microti* (RD1^{mic}, spanning Rv3864 to Rv3876 locus) [45]. Nevertheless, *esat6*-like genes are known to be also present in other non-MTC mycobacteria (Figure 3.2).

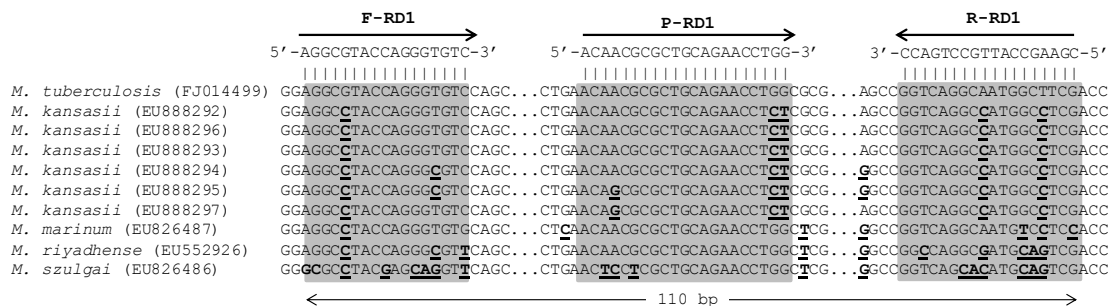


Figure 3.2. Complementary targets of the Rv3875-*esat6* (RD1) targeted probe and flanking primers. Partial alignment of the *esat6* gene sequence of *Mycobacterium tuberculosis* members with other *esat6*-like sequences found in non-MTC species (GenBank accession numbers are indicated inside parenthesis). The complementary targets of the P-*esat6* TaqMan probe and flanking primers (F-*esat6* and R-*esat6*) are indicated (gray boxes). Mismatches in sequences are highlighted in bold and underlined.

The complementary regions of the P-Rv1510, F-Rv1510 and R-Rv1510 probe and flanking primers, respectively, are located in the Rv1510 locus coding a conserved probable membrane protein (as in the sequence with GenBank accession number AL123456, region 1701295 - 1702593), included in the RD4 genomic region, which is present in all MTC members with the exception of *M. bovis* (Figure 3.1).

The complementary regions of the P-Rv2073c, F-Rv2073c and R-Rv2073c probe and flanking primers, respectively, are located in the Rv2073c locus coding a probable short chain dehydrogenase (as in the sequence with GenBank accession number AL123456, region 2330214 - 2330963), included in the RD9 genomic region, which is present in *M. tuberculosis* but absent from *M. bovis* and other MTC species (Figure 3.1). Nevertheless, by performing a BLAST analysis at the GenBank website with the MTC Rv2073c locus sequence, we found putative Rv2073c-like sequences in the publicly available whole genome sequences of other non-MTC mycobacteria (Figure 3.3): *M. kansasii* (GenBank Accession Number CP006835, region 416183 - 416932); *M. avium* subsp. *paratuberculosis* (AE016958, region 1995996 - 1996559); *M. intracellulare* (CP003322, region 2533633 - 2534382); *M. indicus pranii* (CP002275, region 2474147 - 2474896); *M. liflandii* (CP003899, region 2955979 - 2956704); *M. avium* subsp. *hominissuis* (508732639, region 2953585 - 2954334); and *M. marinum* (CP000854, region 3698256 - 3699005).

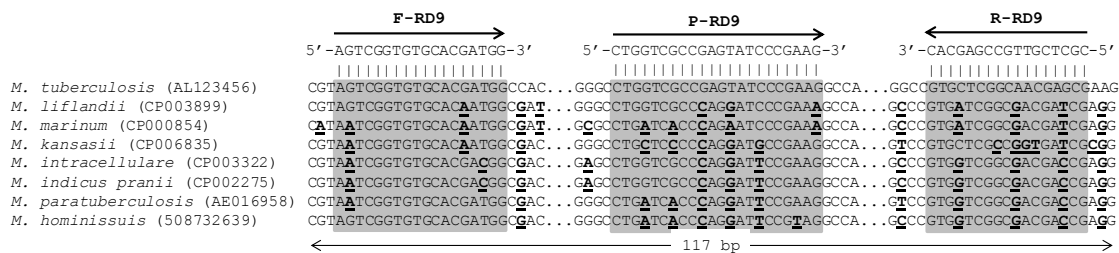


Figure 3.3. Complementary targets of the Rv2073c (RD9) targeted probe and flanking primers. Partial alignment of the Rv2073c locus sequence, located in the RD9 genomic region of *Mycobacterium tuberculosis*, with other potential Rv2073c-like sequences found in other non-MTC species for which whole genome sequences are publicly available (GenBank accession numbers are indicated inside parenthesis). The complementary targets of the P-Rv2073c *TaqMan* probe and flanking primers (F-Rv2073c and R-Rv2073c) are indicated (gray boxes). Mismatches in sequences are highlighted in bold and underlined.

Two-step multiplex real-time PCR algorithm

The amplification-based identification algorithm using DNA extracted from MTC cultures as template consists in two steps: (i) a first duplex real-time PCR targeting the mycobacterial 16S rDNA and the MTC-specific *IS6110*; and (ii) a second triplex real-time PCR targeting the Rv3875-*esat6* (RD1), Rv1510 (RD4) and Rv2073c (RD9) genomic regions of MTC (Figure 3.1). All the amplification reactions were previously optimized using DNA extracted from reference strains as template. Duplex real-time PCR reactions were carried out in a total volume of 20 μ l containing 1 \times SSO Fast Super Mix (Bio-Rad), 0.4 μ M of each primer (F_16SrDNA, R_16SrDNA, F_16SrDNA and R_16SrDNA), 0.15 μ M of each *TaqMan* probe (P_16SrDNA and P_16SrDNA), DNase free water (GIBCO) and 5 μ l of the extracted DNA template. The triplex real-time PCR reactions used 0.25 μ M of F-*esat6*, R-*esat6*, F-Rv1510 and R-Rv1510 primers, 0.4 μ M of F-Rv2073c and R-Rv2073c primers, 0.15 μ M of P-*esat6* and P-Rv1510 *TaqMan* probes, and 0.25 μ M of P-Rv2073c *TaqMan* probe. The thermal cycling conditions were as follows: 1 cycle at 95 $^{\circ}$ C for 2 min, followed by 45 cycles at 95 $^{\circ}$ C for 5 s and 60 $^{\circ}$ C for 10 s. All samples that probed positive for each one of the targets were retested for confirmation. Thermal cycling, fluorescent data collection, and data analysis were performed in a CFX96 (Bio-Rad) detection system real-time PCR instrument, according to the manufacturer's instructions.

Analytical specificity and sensitivity of amplification assays

To investigate whether the probes and respective flanking primers detect their specific targets (16S rDNA, IS6110, Rv3875-*esat6* (RD1), Rv1510 (RD4) and Rv2073c (RD9)), outlined in the two-step multiplex real-time PCR identification algorithm, DNA from MTC and non-MTC isolates (Table 3.1) were used as templates in these assays. To estimate the DNA detection threshold, the duplex and triplex real-time PCR amplification assays were tested with ten-fold diluted DNA reference samples, in concentrations ranging from 1 ng/μl to 1 fg/μl, from *M. bovis*, *M. caprae* and *M. avium* subsp. *avium* strains. These DNA reference samples were obtained and tested in the frame of an European inter-laboratorial ring-test trial organized in 2013 by the European Union Reference Laboratory (EURL) for bovine tuberculosis (VISAVET, Centro de Vigilancia Sanitaria Veterinaria, UCM, Madrid, Spain), in order to assess the sensitivity and specificity of PCR-based detection assays used in National Reference Laboratories (NRLs). For each strain and concentration the PCR assays were performed in triplicate. The end-point corresponded to the dilution at which the assay could detect the respective DNA targets in all three replicates.

Tissue samples and liquid cultures

Lymph nodes, liver, spleen or lung tissue samples collected from 117 animals (69 bovines, 33 wild boars and 15 deer) were used in this work (Table 3.3). Bovine samples were collected from animals clinically suspected of having TB, e.g. presenting a positive reaction in either the single intradermal comparative tuberculin test or the gamma-interferon test, or TB-like lesions detected during routine abattoir inspection, and were submitted to routine control testing under the governmental Portuguese eradication scheme for bovine tuberculosis [18]. TB suspect samples from wild boar and deer sent to INIAV reference laboratories following gross pathological evaluation performed in the field by local veterinarians in hunting activities legally authorized. Samples were submitted during the fourth trimester of 2011 to the pathology and bacteriology laboratories of INIAV and analysed using routine histological and culture-based methods, according to the OIE standard procedures [46]. Tissues selected for bacteriological analysis were homogenized using a pestle and mortar, followed by

decontamination with 4% sodium hydroxide. After neutralization with 10% hydrochloric acid, the macerated suspensions were centrifuged and inoculated into BACTEC 9000 liquid media. Inoculated media were incubated until a positive culture is detected or for a minimum of eight weeks at 37 °C. After incubation, the culture supernatants (either positive or negative) were subjected to a rapid DNA extraction procedure. Briefly, 10 ml of culture was centrifuged at 3800×g for 30 min. The supernatant was discarded and the pellet was washed in 10 ml of PBS and suspended in 250 µl of TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). This suspension was heated in a water bath at 95 °C for 25 min and centrifuged at 15000×g for 5 min. A 150 µl aliquot of the supernatant (containing the DNA) was transferred to a sterile microtube and stored at -20°C until further use. Stock DNA suspensions were diluted ten times in distilled water before its use as template for real-time PCR assays. DNA extracted solutions from positive cultures were used for species identification of presumptive mycobacteria isolates as routinely performed at our reference lab, using the commercial reverse hybridization assays INNO-LiPA Mycobacteria V2 (Innogenetics, Belgium) or GenoType *Mycobacterium* CM/AS (Hain diagnostics, Germany), following the manufacturer's instructions, and the restriction endonuclease analysis with *RsaI* and *SacII* of the PCR-amplified *gyrB* gene (for presumptive MTC isolates) [21]. Additionally, DNA extracted solutions from both positive and negative cultures were used for assessing the performance of the developed *TaqMan*®-based real-time PCR identification algorithm.

Table 3.3. Results of the application of the duplex (16S rDNA and IS6110) and triplex (Rv3875-RD1, Rv1510-RD4 and Rv2073c-RD9) real-time PCR amplification assays to DNA templates extracted from BACTEC liquid cultures of TB-suspected animal tissues.

Origin of tissues	Presence of lesions ¹	Bacteriological analysis ²	Sample ID	Number of samples	16S rDNA ³	IS6110 ³	RD1 ³	RD4 ³	RD9 ³
Bovine	+	<i>M. bovis</i>	30101; 30221; 30496; 31785; 33758; 35716; 36632; 37722; 42089; 30504/11; 30504/12; 30504/14; 30504/2; 30504/3; 30504/6; 30504/7; 31719/2; 36867/10; 36867/13; 36867/4; 37720/1; 41679/1	22	+	+	+	-	-
	-	<i>M. bovis</i>	30504/4; 30504/5; 36867/7	3	+	+	+	-	-
	+	Non-MTC	35690	1	+	-	-	-	-
	-	Non-MTC	37707/2; 31389	2	+	-	-	-	-
	+	-	41680/1; 31784	2	-	-	-	-	-
	-	-	31093; 31094; 31388; 31786; 32079; 32451; 33584; 33716; 33757; 33787; 33788; 36835; 37721; 40038; 40336; 40849; 40853; 40854; 41542; 41656; 41678; 30504/1; 30504/10; 30504/8; 30504/9; 31717/1; 31717/2; 31719/1; 36867/20; 37707/4; 37707/6; 37720/2; 39850/2; 39850/3; 40037/1; 40056/2; 40056/6; 41679/2; 41680/3	39	-	-	-	-	-

Table 3.3. (cont.)

Origin of tissues	Presence of lesions ¹	Bacteriological analysis ²	Sample ID	Number of samples	16S rDNA ³	IS6110 ³	RD1 ³	RD4 ³	RD9 ³
Wild boar	+	<i>M. bovis</i>	37481/1; 37481/2; 37481/3; 37481/4; 37481/5; 37708/1; 37708/3; 37708/2; 37708/4; 41674/1; 41675/3; 41677/2; 41677/6; 41677/7; 41677/8; 41963/10; 41963/2; 41963/7	18	+	+	+	-	-
	+	Non-MTC	33524/2; 35732	2	+	+	+	-	-
	+	Non-MTC	38951/5; 37548	2	+	-	-	-	-
	-	Non-MTC	37549/2; 37549/3; 41677/9	3	+	-	-	-	-
	+	-	33760; 33763/1; 33763/2; 41675/2	4	-	-	-	-	-
	-	-	33524/1; 33524/3; 37549/1; 41963/4	4	-	-	-	-	-
Deer	+	<i>M. bovis</i>	33761; 34827; 37486; 38534; 34920/1; 34920/2; 34920/3; 34920/4; 35730/2; 35733/1; 41676/7; 41964/2	12	+	+	+	-	-
	-	-	34917; 41676/5; 41964/1	3	-	-	-	-	-

¹Presence of lesions compatible with tuberculosis; ²Detection of *M. bovis* or other non-MTC mycobacteria by culture of tissue samples;

³Amplification (+) or no amplification (-).

Statistics

For the computation of the kappa coefficient, for measuring the agreement between the results of the routinely performed bacteriological culture procedures, including the respective molecular identification of the isolates, and the real-time PCR identification algorithm herein described, all BACTEC cultures were used ($n = 117$). The kappa coefficient, with confidence intervals, was computed using the clinical research calculators of the online VassarStats software (<http://vassarstats.net>).

3.4. Results

Design of the identification algorithm

A two-step identification algorithm, comprising two sequential *TaqMan*-based multiplex real-time PCR reactions, was implemented for the identification of veterinary-relevant MTC isolates (Figure 3.1). The assays use DNA extracted from mycobacterial cultures as template. The first step allows the identification of the cultures as MTC members, by targeting their *IS6110* element, or as a mycobacterial species, if only a 16S rDNA product is detected in the duplex amplification reaction (Figure 3.1). If a MTC member is identified, the second amplification step allows to assess the presence or absence of the *Rv3875-esat6* (RD1), *Rv1510* (RD4) and *Rv2073c* (RD9) loci in the respective genome. The correspondent pattern allows to infer the species of the isolate as *M. tuberculosis*, if all loci are present, as *M. caprae*, if only the *Rv3875-esat6* (RD1) and *Rv1510* (RD4) loci are present, as *M. bovis*, if only *Rv3875* (RD1) locus is present, and as *M. bovis* BCG if all the previous loci are absent (Figure 3.1; see discussion below).

Analytical specificity and sensitivity of the identification algorithm

The algorithm was tested using DNA extracted from previously identified MTC and non-MTC reference and clinical strains as template (Table 3.1; Figure 3.4). In the first step, the target 16S rDNA region was only detected in mycobacterial species and the *IS6110* element was only detected in MTC species (Table 3.1). In the second step, the *Rv3875-esat6* (RD1) locus region was detected in all pathogenic MTC strains, the

Rv1510 (RD4) region was detected only in *M. tuberculosis*, *M. africanum* and *M. caprae* strains, and the Rv2073c (RD9) region was only detected in *M. tuberculosis* strains (Table 3.1). None of these genomic regions were detected in vaccine *M. bovis* BCG strains. The results of the analytical sensitivity were as follows:

The first duplex reaction could detect the presence of *M. bovis* until the dilution containing 1 pg/μl (Ct ≈ 35) of DNA template, with both the 16S rDNA and IS6110 targets (however, at least one of the three replicates yielded positive amplification results further until the dilution containing 10 fg/μl of template DNA). The same detection limit of 1 pg/μl was found for the following triplex reaction targeting the Rv3875-*esat6* (RD1) of *M. bovis*, and the Rv1510 (RD4) and Rv2073c (RD9) loci were not detected, as expected.

The first duplex reaction detected the presence of *M. caprae* until the dilution containing 1 pg/μl (Ct ≈ 34) and 0.1 pg/μl (Ct ≈ 39) of DNA template, with the 16S rDNA and IS6110 targets, respectively. However, at least one of the three triplicates yielded positive amplification results further until the dilution containing 1 and 10 fg/μl for the 16S rDNA and IS6110 targets, respectively. The following triplex reaction showed a detection limit of 10 pg/μl (Ct ≈ 37) of DNA template for both Rv3875-*esat6* (RD1) and Rv1510 (RD4) targets, and Rv2073c (RD9) was not detected, as expected.

Finally, also as expected, *M. avium* subsp. *avium* was only detected in the first duplex reaction with the mycobacterial 16S rDNA-targeted probe, with a detection limit of 1 pg/μl (Ct ≈ 35) of DNA template (however, at least one of the three triplicates yielded positive amplification results further until the dilution containing 1 fg/μl of template DNA).

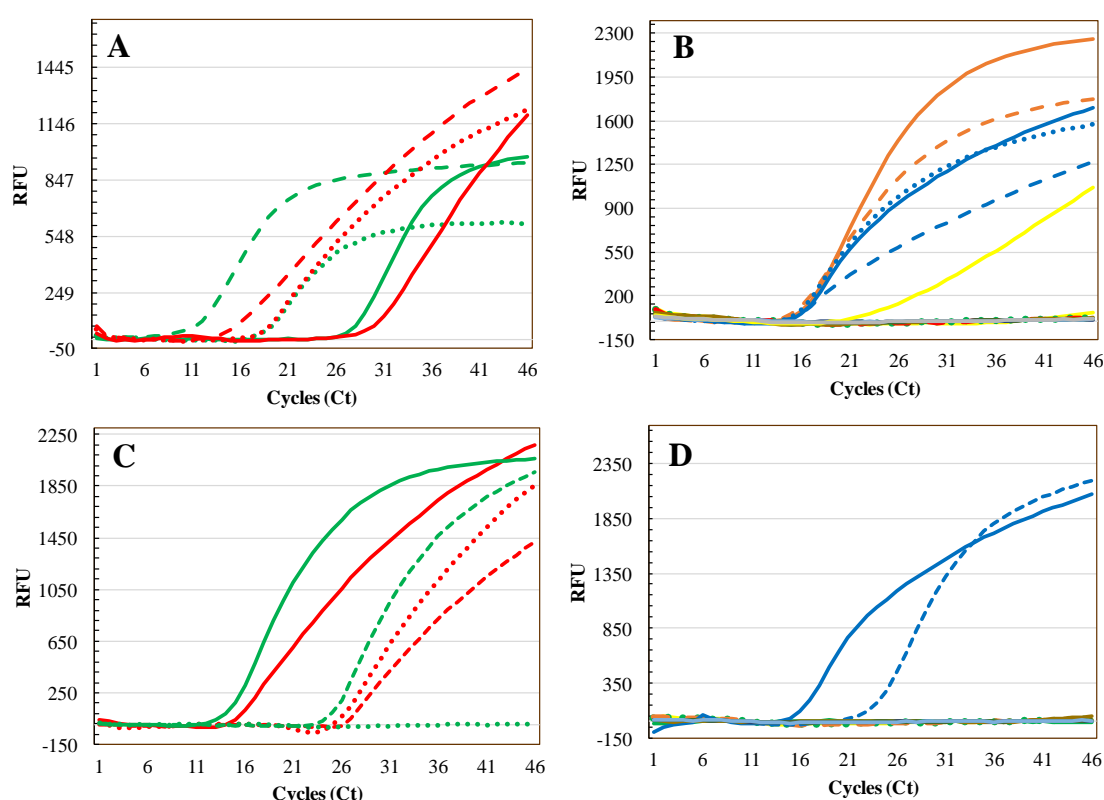


Figure 3.4. Illustration of the real-time PCR amplification curves obtained. 16S rDNA and IS6110-targeted duplex (left) and Rv3875-*esat6* (RD1), Rv1510 (RD4) and Rv2073c (RD9) targeted triplex (right) real-time PCR assays for strains *M. tuberculosis* H37Ra (continuous line), *M. caprae* LNIV17320 (dashed line) and *M. bovis* LNIV13027 (dotted line) (A, B) and for culture samples 37481/5 (continuous line), 37708/1 (dashed line) and 38951/5 (dotted line) (C, D). Genomic targets curves colours: red (16S rDNA), green (IS6110), RD1 (blue), RD4 (orange) and RD9 (yellow).

Application of the identification algorithm to BACTEC cultures of veterinary origin

The identification algorithm was tested using DNA extracted from a set of 117 sequential liquid cultures, using a simple boiling method, obtained under the routine testing of TB-suspected tissue samples originally collected from bovines (n = 69), deer (n = 15) and wild boar (n = 33) (Table 3.3; Figure 3.4). Fifty five cultures were identified as *M. bovis* by the routine bacteriological analysis and 10 cultures were identified as non-MTC species: *M. avium* (n = 5), *M. scrofulaceum* (n = 2) and *Mycobacterium* spp. (n = 3). No bacterial growth was detected in 52 samples. *Mycobacterium caprae* was not identified from any sample. The identification

algorithm developed, based in the duplex (16S rDNA and IS6110) and triplex (Rv3875-RD1, Rv1510-RD4 and Rv2073c-RD9) real-time PCR amplification assays, presented an almost perfect agreement with the results of the routine bacteriological analysis (Table 3), with a kappa coefficient of 0.970 (CI_{P95%} 0.929-1.000).

All 55 *M. bovis* liquid cultures yielded amplification results for Rv3875-*esat6* (RD1) but Rv1510 (RD4) and Rv2073c (RD9) regions were absent. No amplification was detected for any of the 16S rDNA, IS6110, RD1, RD4 and RD9 genomic targets in the 52 negative samples.

Eight cultures were identified as non-MTC species by routine bacteriology analysis: *M. scrofulaceum* (31389); *M. avium* (37549/2, 37549/3, 37707/2, 37548 and 38951/5); and *Mycobacterium* spp. (35690 and 41677/9). All these cultures yielded a positive amplification result only for the mycobacterial 16S rDNA region when tested with the real-time PCR-based identification algorithm (Table 3.3). However, two additional cultures from wild boar were identified as non-MTC species by routine bacteriology, *M. scrofulaceum* (33524/2) and *Mycobacterium* sp. (35732), using the GenoType CM/AS *Mycobacterium* identification assay, but yielded positive amplification results for the IS6110 element and Rv3875-*esat6* (RD1) locus, compatible with the *M. bovis* RDs profile (Table 3.3). These two BACTEC cultures were obtained from wild boar tissues samples presenting lesions compatible with tuberculosis when analysed by histology (Table 3.3).

3.5. Discussion

In this work we aimed to implement a fast and straightforward molecular assay to identify the most relevant MTC members causing disease in domestic (mainly livestock) and big game animals, such as wild boars and deer, commonly received in our national reference lab for TB laboratory diagnosis. The MTC comprises a group of closely-related TB-associated species, currently including: *M. tuberculosis* (the predominant cause of human TB); "*M. canettii*" (a very rare MTC biotype); *M. africanum* (mainly associated to human TB in Africa); *M. pinnipedii* (the cause of endemic TB in several seal species); *M. microti* (with bank voles and other small rodents as natural hosts, rarely identified from other mammals); *M. mungi* (associated to

TB in banded mongooses in Botswana); *M. orygis* (a recently described and less known rare species, with oryxes, waterbucks, and gazelles as potential hosts in Africa, and bovines and rhesus monkeys in South Asia); and *M. bovis* and *M. caprae* (the worldwide predominant cause of bovine and goat TB, respectively, but also causing disease in a wide range of domestic and wild animals, including humans) [5, 6, 8-10, 47, 48]. In our lab, more than 38% of the samples from suspect cattle previously analysed between 2002 and 2010 were confirmed as TB positive and 92% and 1% of the mycobacterial isolates were identified as *M. bovis* and *M. caprae*, respectively [18]. The remaining isolates belonged to *Mycobacterium avium* complex or to other environmental nontuberculous mycobacteria. No other of the above referred MTC species were ever isolated in our lab, with the exception of a few *M. tuberculosis* isolates collected from zoo captive mandrills and a dog [49, 50]. Although a rare event, infection with *M. tuberculosis* mostly occurs in animals living in close contact with humans, such as pets, pigs, cattle and captive animals [51-57], and reference veterinary diagnostic labs should be aware for these infections as they raise important public health concerns. Taking into account the above mentioned considerations, we selected *M. bovis*, *M. caprae* and *M. tuberculosis* as the relevant target species for the implementation of our real-time PCR-based identification assay.

The proposed identification assay involves a first amplification step where the tested isolate will be assigned as an MTC member (by detecting the presence of the MTC-specific IS6110 element), which is the most relevant case, or, alternatively, as a non-MTC *Mycobacterium* species. It has recently been found that IS6110-like elements may be present in other non-MTC mycobacteria such as *M. smegmatis* [58]. However, the probe and respective flanking primers used show no relevant complementary regions with these IS6110-like nucleotide sequences [19].

If an MTC isolate is found, the subsequent RDs-targeted triplex real-time PCR assay allow the identification to the species level as *M. bovis*, *M. bovis* BCG, *M. caprae* or *M. tuberculosis*, according to their distinct patterns of presence or absence of Rv3875-*esat6* (RD1), Rv1510 (RD4) and Rv2073c (RD9) loci (Figure 3.1). We decided to target these LSPs (RD1, RD4 and RD9) instead of SNPs (e.g. in *gyrB* gene) to discriminate between these MTC members since the PCR-based detection assays to differentiate SNPs may

be less robust and accurate, requiring a much more strict control of the experimental conditions to maintain the specificity.

All MTC members, with the exception of *M. bovis* BCG (and also *M. mungi* and *M. microti*), harbour an intact RD1 genomic region, which include the *esat6* gene that should be detected by our assay (Figure 3.1). It is widely accepted that the vaccine strain *M. bovis* BCG is not pathogenic in part due to the loss of this RD1 region, which include the genes *esat6* and *cfp10* essential for pathogenesis in *M. tuberculosis* being two of the most important virulence factors of this pathogen [8, 34]. Noteworthy, these genes (and homologous RD1-like genomic regions) were also found among nontuberculous mycobacteria such as *M. kansasii*, *M. szulgai*, *M. marinum* and *M. riyadhense* [59, 60]. Several of these non-MTC mycobacteria can be also found infecting domestic and wild animals [61]. However, as shown in Figure 3.2, the several mismatches existing in the complementary regions of our RD1-targeted primers and/or *TaqMan* probe should be sufficient to prevent the amplification and detection of the RD1-like sequences of the rare pathogens *M. szulgai* and *M. riyadhense*. Although *Mycobacterium kansasii* and *M. marinum* present less mismatches in the respective primers/probe homologous complementary targets (Figure 3.2), and could be potentially yield weak false-positive results in sub-optimized amplification assays, these pathogens are rarely found infecting domestic and wild mammal species [61] and they lack the *IS6110* element, therefore previously excluded in our algorithm .

The RD4 genomic region is considered to be present in all MTC members with the exception of *M. bovis* (and of *M. bovis* BCG). In fact, the detection of an RD4 deletion has been used as a major criterion for distinguishing the major veterinary relevant MTC species *M. bovis* from *M. caprae* [2, 26, 28, 30, 35]. However, a recent study reported that RD4 may be also absent, at least in part, from some *M. caprae* isolates in Germany, which corresponded to their respective geographic origins and genotypes [62]. These authors found that, from the twelve *M. caprae* isolates they have studied, four lacked a 5 kb region in the left part of the RD4 region, which they called the Karwendel type, and another four isolates presented a 38 kb deletion, encompassing the whole RD4, which they called the Lechtal type. Four additional isolates presented the usual and apparently complete RD4 region (Allgäu type). Apparently, if tested with our RD4 (Rv1510 locus)-targeted real-time PCR assay, only the Karwendel and Allgäu types of

M. caprae would yield a positive amplification result and the Lechtal type would be misidentified as *M. bovis* [62]. All five Portuguese *M. caprae* isolates tested in this work were RD4 (Rv1510 locus) positive (Table 3.1) and it is also described that this genomic region was detected in the majority of the studied Spanish isolates [63]. Therefore, we sustain that the RD4 genomic target, namely the Rv1510 locus, is a useful marker allowing the discrimination of the majority of *M. caprae* clinical isolates from *M. bovis*. However, more studies need to be performed in a wider collection of *M. caprae* isolates, with an extended range of geographical origins, in order to better assess the structure of this RD4 genomic region and, if necessary, to update our identification algorithm with alternative genomic markers for the effective differentiation of *M. caprae* from *M. bovis*.

The RD9 genomic region is considered to be only present in *M. tuberculosis* (and in the rarely found taxa “*M. canettii*”), being absent from all other MTC species. We have found potential Rv2073c-like sequences in other non-MTC *Mycobacterium* species after a BLAST analysis on the respective whole genome sequences available in GenBank, namely in *M. liflandii* (a frog pathogen), *M. marinum*, *M. kansasii*, *M. intracellulare*, *M. indicus pranii* (a non-pathogenic saprophytic organism), *M. avium* subsp. *paratuberculosis* and *M. avium* subsp. *hominissuis* (Figure 3.3). Nevertheless, the several mismatches present in the complementary regions of these species for our RD9-targeting probe and flanking primers will prevent any amplification, and consequent potential false-positive detection by our real-time PCR assays.

In our identification algorithm, *M. tuberculosis* isolates will present an Rv3875-*esat6* (RD1), Rv1510 (RD4) and Rv2073c (RD9) loci amplification positive profile. “*Mycobacterium canettii*” presents the same profile but this species is very rarely found and, as far as we know, was never isolated from animal species. *Mycobacterium bovis* isolates will also present a distinct profile, with Rv3875-*esat6* (RD1) amplification positive and Rv1510 (RD4) and Rv2073c (RD9) negative (*M. bovis* BCG isolates will lack the RD1 region). Finally, *M. caprae* isolates will harbour the profile Rv3875 (RD1) and Rv1510 (RD4) positive and Rv2073c (RD9) negative. However, other MTC species will also present this profile, namely *M. africanum*, *M. pinnipedii* and *M. orygis* [8]. Regarding the infection of animals by the predominantly human pathogen *M. africanum*, at least in some cases, this species may have been misidentified and might

represent the recently described *M. orygis* instead [10]. Nonetheless, as also mentioned at the beginning of this section, all these MTC species are rarely found in domestic (particularly livestock) and big game animals, such as deer and wild boar that are usually received in our lab for TB testing. Therefore, we sustain that the amplification profile RD1 and RD4 positive and RD9 negative is useful for the identification of *M. caprae* isolates in the context of a veterinary diagnostic lab.

The specificities of the proposed duplex and triplex real-time PCR assays were evaluated against a panel of 76 MTC, non-MTC mycobacteria and other bacterial species (Table 3.1). All assays were 100% specific for the respective target organisms (as assessed by the amplification profiles of the 16S rDNA, IS6110, RD1, RD4 and RD9 genomic targets). As expected, tested *M. caprae* and *M. africanum* strains harboured an identical amplification profile. Additionally, 117 BACTEC positive and negative liquid cultures were tested, resulting in an almost perfect agreement with the results of the routine bacteriological analysis (Table 3.3). No amplification results were obtained for the 52 BACTEC culture negative samples, similarly to the negative controls of amplification, also demonstrating that no cross-contaminations and no amplification artefacts were produced during execution of the assays.

Only two discordant cases were found among the results of our identification algorithm and the routine bacteriology procedures currently used in our lab (Table 3.3): wild boar samples 33524/2 and 35732 yielded non-MTC cultures further identified as *M. scrofulaceum* and *Mycobacterium* sp., respectively. However, the RDs amplification profiles were concordant with *M. bovis* when DNA extracted from these cultures were used as template. The tissue samples used to inoculate these liquid cultures presented TB-compatible lesions. Additionally, the presence of MTC was confirmed in these samples by an IS6110-targeted semi-nested real-time PCR approach that we also recently described [19] (data not shown). Therefore, although TB-like lesions identified by histopathology can be induced by other bacteria or non-MTC mycobacteria, the IS6110-specific positive amplification results suggest that MTC members were most probably associated with the observed lesions. The detection of an *M. bovis* RDs profile by our identification algorithm in samples 33524/2 and 35732 probably reflects that they harboured a mixed culture of this species with other non-MTC fast-growing mycobacteria. These may potentially overgrow any *M. bovis* isolate present in the tissue

samples and mask its presence, being its vestigial template DNA eventually only detected by the sensitive real-time PCR amplification assays [19, 64].

In this work we describe a rapid and robust five-target *TaqMan*[®]-based real-time PCR identification algorithm, based on genomic deletion analysis, to identify the MTC species most commonly associated with TB in livestock and other animals. The assay is significantly faster and simpler than other SNPs-targeted restriction analysis or reverse line probe hybridization-based molecular assays, since it bypasses any post-amplification experimental steps and can be used with DNA templates extracted directly from liquid cultures using a simple, low-cost and fast boiling-based method. The assay is able to be further automated and implemented in the routine diagnostics framework of veterinary diagnostics laboratories, with a particular focus for reference laboratories.

3.6. References

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CHAPTER 4

Identification of *Mycobacterium tuberculosis* and *M. bovis* using duplex Loop-Mediated Isothermal DNA Amplification (dLAMP) and colorimetric lateral flow devices

Identification of *Mycobacterium tuberculosis* and *M. bovis* using duplex Loop-Mediated Isothermal DNA Amplification (dLAMP) and colorimetric lateral flow devices

4.1. Summary

Rapid, user-friendly and affordable diagnostic tests for use in the point-of-decision or point-of-care settings are in high demand globally. Species included in the *Mycobacterium tuberculosis* complex (MTC) are responsible for tuberculosis in both animals and humans. Detection of MTC members is generally performed by cumbersome conventional culture-based methods, which usually takes up to eight weeks. Molecular diagnosis strategies were also developed but most of these requires skilled operators and sophisticated equipments and facilities. More recently, the Loop-Mediated Isothermal Amplification (LAMP) technique showed promise for the development of rapid, low-cost, sensitive and specific tests for detecting pathogens. In this work we optimized two duplex LAMP (dLAMP) assays for the identification of MTC and *Mycobacterium tuberculosis*, and MTC and *M. bovis*, presenting similar sensitivities and specificities when compared to standard PCR approaches. The amplification results are assessed colorimetrically by using simple and commercially available nucleic acid lateral flow (NALF) strips. All the identification process takes around 70 minutes.

4.2. Introduction

The demand for rapid point-of-decision diagnostics for infectious diseases has been steadily increasing globally, namely in veterinary medicine [1]. Tuberculosis (TB), caused by *Mycobacterium tuberculosis*, *M. bovis* and other closely related species of the *M. tuberculosis* complex (MTC), is a major infectious disease of humans, and domestic and wildlife animals across the World [2-4]. The laboratorial detection of MTC members is generally performed by cumbersome conventional culture-based methods, which usually takes up to eight weeks. Nevertheless, molecular diagnostic tests play an important role in detecting MTC members and in monitoring the spread of tuberculosis

in both humans and animals. In the case of animal TB, most described molecular detection and identification tests have bovine tuberculosis, and the respective causal agents, as target. Most assays are PCR or real-time PCR-based, allowing a rapid and highly sensitive and specific laboratorial assessment of TB. However, poorly equipped laboratories in low-resource settings face major difficulties for accessing these molecular techniques, that are reliant upon expensive, sophisticated and relatively fragile equipments. A new group of nucleic acid detection assays that exploit isothermal amplification mechanisms have been developed as potential diagnostic tools for use in either the field or in low-cost laboratory settings [5-7]. One most promising technique is the loop mediated isothermal amplification (LAMP), which is a relatively novel approach, and allows nucleic acid amplification with high specificity, sensitivity and rapidity under isothermal conditions [8]. The method was originally described by Notomi and colleagues in 2000 [8] and have been used for the detection of a wide range of viruses, parasites and bacteria [9-14]. The method relies on an autocycling strand displacement DNA synthesis by the *Bst* DNA polymerase large fragment that is a DNA polymerase with high strand displacement activity, superseding thermal denaturation steps [8]. Therefore, a thermal cycler is not needed, and the reaction can be performed with a simple and inexpensive thermostatic water bath or a heating block. LAMP also requires a set of a minimum of four primers: two inner primers (forward inner primer - FIP, and the backward inner primer - BIP), and two outer primers (F3/B3). Additional loop primers can be used, which usually speed up the reaction [15]. LAMP is highly specific for the target sequence since it requires the recognition of at least six distinct complementary nucleotide segments of the target DNA. The amplification efficiency of the LAMP method is extremely high, it can amplify 10^9 copies of nucleic acid target, usually at a temperature between 60-65 °C, within 30-60 min [16, 17]. The amplification and identity of the LAMP products can be confirmed by conventional agarose gel electrophoresis, by visual inspection of the increase in turbidity, or colour change, of the reaction mixture, by the use of spectrophotometric equipments to measure the increase of turbidity or by using intercalating fluorescent dyes [18]. Equipment-free methods for unambiguous detection and identification of LAMP products would increase the feasibility of using LAMP for detection of mycobacteria outside the laboratory. One promising approach is the use of lateral-flow devices

(LFDs) for the detection of labels incorporated into the amplification products, a technique sometimes referred to as nucleic acid lateral flow (NALF). Tests in LFD format have a number of advantages for use in the field, and specific LFD immunoassays have been extremely successful in areas of point-of-care and on-site testing [19-23]. Generic commercially available LFD strips can be used for this purpose. These chromatographic strips can detect biotin-labelled DNA fragments hybridized with complementary FITC-labelled probes. FITC is detected in the strips by the formation of complexes with gold-conjugated anti-FITC antibodies. In this work we combined the advantages of both LAMP and LFD technologies to implement a simple, rapid, sensitive and specific molecular assay to identify MTC members, particularly the most relevant *M. tuberculosis* and *M. bovis* species, suitable for use in low-resource laboratory settings.

4.3. Materials and Methods

Bacterial strains and DNA extraction

Reference strains and previously identified clinical isolates of MTC, non-MTC mycobacteria and non-mycobacterial species, maintained at the Portuguese reference laboratory for animal diseases (*Instituto Nacional de Investigação Agrária e Veterinária*, INIAV, I.P.), were used for optimization of amplification assays (Table 4.1). MTC isolates were previously identified to the species level by PCR-restriction endonuclease analysis of the *gyrB* gene [24-26].

Table 4.1. Reference and clinical bacterial strains used to assess the specificity of the dLAMP-LFD assays and respective amplification results.

Species	Strains	IS6110	RD4 flanking	RD9
<i>M. tuberculosis</i>	ATCC 25177; LNIV 9605	+	-	+
<i>M. caprae</i>	LNIV 17320; 4958/0/05; 8403; 15244; and 20752	+	-	-
<i>M. bovis</i>	AN5; LNIV 13027; 5530/0/05; 11265; 7230/4; 14421/2; 24497/6; 8855; 5889; 10044; 14577; 13280/6; 13280/4; 34875; and 20564	+	+	-
<i>M. bovis</i> BCG	ATCC 27291	+	+	-
<i>M. avium</i> subsp. <i>avium</i>	ATCC 25291	-	-	-
<i>M. avium</i> subsp. <i>hominissuis</i>	LNIV 23063/4; IHMT 31/06; 229/06; 193/08; 189/05; 8/05; 291/05; 386/08; 92/06; 78/06; 327/07; 73/11; 436/05; and 68/06	-	-	-
<i>M. avium</i> subsp. <i>paratuberculosis</i>	LNIV 39888	-	-	-
<i>M. scrofulaceum</i>	LNIV 31389	-	-	-
<i>Acinetobacter baumannii</i>	LNIV 1628/12	-	-	-
<i>Arcanobacterium pyogenes</i>	VLA 1321	-	-	-
<i>Bacillus licheniformis</i>	VLA 1831	-	-	-
<i>Corynebacterium striatum</i>	LNIV 12352	-	-	-
<i>Enterobacter amnigenus</i>	LNIV 6050/II	-	-	-
<i>Klebsiella pneumonia</i>	VLA 1643	-	-	-
<i>Listeria monocytogenes</i>	VLA 1774	-	-	-
<i>Proteus mirabilis</i>	LNIV 2269/II	-	-	-
<i>Pseudomonas aeruginosa</i>	VLA 67	-	-	-
<i>Salmonella</i> Dublin	VLA 1272	-	-	-
<i>Staphylococcus aureus</i>	VLA 1032	-	-	-
<i>Streptococcus agalactiae</i>	VLA 33	-	-	-
<i>Yersinia enterocolitica</i>	VLA 1884	-	-	-

ATCC, American Type Culture Collection, USA; LNIV, *Laboratório Nacional de Investigação Veterinária* (currently INIAV, I.P.), Lisbon, Portugal; IHMT, *Instituto de*

Higiene e Medicina Tropical, Lisbon, Portugal; VLA, Veterinary Laboratory Agency, UK; Amplification (+) or no amplification (-).

DNA extraction from cultures grown in liquid media was achieved by a combined bead beating and enzymatic extraction method described elsewhere [27]. Briefly, 0.5 ml bacterial culture were washed in PBS buffer and heat inactivated at 100 °C for 15 min. Tubes containing zirconium beads were used to resuspend the culture pellet in lysis buffer (0.4 M NaCl, 40 mM Tris-HCl, pH 8, 2 mM EDTA, 0.6% SDS, 0.034 mg/ml proteinase K) for mechanical disruption in the FastPrep FP120 Bio101 (Savant Instruments, Inc., Holbrook, NY) at 6.5 ms⁻¹ for 45 s, and then incubated overnight at 37 °C, followed by standard phenol–chloroform purification and ethanol precipitation of DNA. DNA concentration and purity were estimated by measuring the absorbance at 260 nm (A₂₆₀) and by A₂₆₀/A₂₈₀ and A₂₆₀/A₂₃₀ ratios, respectively, using a NanoDrop 1000 spectrophotometer (NanoDrop). Genomic DNA suspensions were stored at - 20 °C until further use. Furthermore, a group of 50 DNA samples extracted from MTC strains, previously isolated from sputum human samples, were also supplied by *Instituto de Higiene e Medicina Tropical* (IHMT) (Table 4.2).

Table 4.2. Results of the dLAMP-LFD #A assay tested with DNA templates extracted from human isolates.

Origin of tissues	Bacteriological analysis	Sample ID	Number of samples	IS6110 ¹	RD9 ¹
Human	<i>M. tuberculosis</i>	IHMT 1; IHMT 2; IHMT 3; IHMT4; IHMT 5; IHMT 6; IHMT 7; IHMT 8; IHMT 9; IHMT 10; IHMT 11; IHMT 12; IHMT 13; IHMT 14; IHMT 15; IHMT 16; IHMT 17; IHMT 18; IHMT 19; IHMT 20; IHMT 21; IHMT 22; IHMT 23; IHMT 24; IHMT 25; IHMT 26; IHMT 27; IHMT 28; IHMT 29; IHMT 30; IHMT 31; IHMT 32; IHMT 35; IHMT 38; IHMT 40; IHMT 41; IHMT 42; IHMT 43; IHMT 44; IHMT 45	40	+	+
	<i>M. bovis</i> BCG	IHMT 33; IHMT 34; IHMT 36; IHMT 37; IHMT 48; IHMT 50	6	+	-
	<i>M. africanum</i>	IHMT 39; IHMT 46; IHMT 47; IHMT 49	4	+	-

¹Amplification (+) or no amplification (-).

Animal tissue samples, liquid cultures and rapid DNA extraction

Lymph nodes, liver, spleen or lung tissue samples collected from 117 animals (69 bovines, 33 wild boars and 15 deer) were used in this work (Table 4.3). Bovine samples were submitted to routine control testing in our reference lab under the governmental Portuguese eradication scheme for bovine tuberculosis [28]. Wild boar and deer samples were sent to our lab following gross pathological evaluation performed in the field by local veterinarians in hunting activities. Samples were submitted during the fourth trimester of 2011 and analysed using routine histological and culture-based methods, according to the OIE standard procedures [29]. Tissues selected for bacteriological analysis were homogenized using a pestle and mortar, followed by decontamination with 4% sodium hydroxide. After neutralization with 10% hydrochloric acid, the macerated suspensions were centrifuged and inoculated into BACTEC 9000 liquid media. Inoculated media were incubated until a positive culture is detected or for a minimum of eight weeks at 37 °C. After incubation of the inoculated media, the culture supernatants (either positive or negative) were subjected to a rapid DNA extraction procedure. Briefly, 10 ml of culture was centrifuged at 3800 ×g for 30 min. The supernatant was discarded and the pellet was washed in 10 ml of PBS and suspended in 250 µl of TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). This suspension was heated in a water bath at 95 °C for 25 min and centrifuged at 15000 ×g for 5 min. A 150 µl aliquot of the supernatant (containing the DNA) was transferred to a sterile microtube and stored at -20°C until further use. Stock DNA suspensions were diluted ten times in distilled water before its use as template for LAMP reactions. DNA extracted solutions from positive cultures were used for species identification of presumptive mycobacteria isolates as routinely performed at our reference lab, using the commercial reverse hybridization assays INNO-LiPA Mycobacteria V2 (Innogenetics, Belgium) or GenoType *Mycobacterium* CM/AS (Hain diagnostics, Germany), following the manufacturer's instructions, and the restriction endonuclease analysis with *RsaI* and *SacII* of the PCR-amplified *gyrB* gene (for presumptive MTC isolates) [30]. Additionally, DNA extracted solutions from both positive and negative BACTEC liquid cultures were used for assessing the performance of the developed duplex LAMP coupled to a nucleic acid lateral flow dipstick strategy for assessing the results (dLAMP-LFD).

Table 4.3. Results of the dLAMP-LFD #B assay tested with DNA templates extracted from isolates from TB-suspected animal tissues

Origin of tissues	Presence of lesions ¹	Bacteriological analysis ²	Sample ID	Number of samples	IS6110 ³	RD4 flanking ³
Bovine	+	<i>M. bovis</i>	30101; 30221; 30496; 31785; 33758; 35716; 36632; 37722; 42089; 30504/11; 30504/12; 30504/14; 30504/2; 30504/3; 30504/6; 30504/7; 31719/2; 36867/10; 36867/13; 36867/4; 37720/1; 41679/1	22	+	+
	-	<i>M. bovis</i>	30504/4; 30504/5; 36867/7	3	+	+
	+	Non-MTC	35690	1	-	-
	-	Non-MTC	37707/2; 31389	2	-	-
	+	-	41680/1; 31784	2	-	-
	-	-	31093; 31094; 31388; 31786; 32079; 32451; 33584; 33716; 33757; 33787; 33788; 36835; 37721; 40038; 40336; 40849; 40853; 40854; 41542; 41656; 41678; 30504/1; 30504/10; 30504/8; 30504/9; 31717/1; 31717/2; 31719/1; 36867/20; 37707/4; 37707/6; 37720/2; 39850/2; 39850/3; 40037/1; 40056/2; 40056/6; 41679/2; 41680/3	39	-	-

Table 4.3. (Cont.)

Origin of tissues	Presence of lesions ¹	Bacteriological analysis ²	Sample ID	Number of samples	IS6110 ³	RD4 flanking ³
Wild boar	+	<i>M. bovis</i>	37481/1; 37481/2; 37481/3; 37481/4; 37481/5; 37708/1; 37708/3; 37708/2; 37708/4; 41674/1; 41675/3; 41677/2; 41677/6; 41677/7; 41677/8; 41963/10; 41963/2; 41963/7	18	+	+
	+	Non-MTC	33524/2; 37548; 35732	3	-	-
	+	Non-MTC	38951/5	1	-	-
	-	Non-MTC	37549/2; 37549/3; 41677/9	3	-	-
	+	-	33760; 33763/1; 33763/2; 41675/2	4	-	-
	-	-	33524/1; 33524/3; 37549/1; 41963/4	4	-	-
Deer	+	<i>M. bovis</i>	33761; 34827; 37486; 38534; 34920/1; 34920/2; 34920/3; 34920/4; 35730/2; 35733/1; 41676/7; 41964/2	12	+	+
	-	-	34917; 41676/5; 41964/1	3	-	-

¹Presence of lesions compatible with tuberculosis; ²Detection of *M. bovis* or other non-MTC mycobacteria by culture of tissue samples;

³Amplification (+) or no amplification (-)

Design of LAMP primers and LFD probes

Sequences of the *IS6110* and of the Regions of Difference 4 (RD4, including the *Rv1510* locus, absent from *M. bovis*) and 9 (RD9, including the *Rv2073c* locus, only present in *M. tuberculosis*) from MTC members were retrieved from NCBI-GenBank. Two sets of six LAMP primers, including loop primers, were designed, one to target the *IS6110* element of MTC species and a second one to target the RD9 present in *M. tuberculosis* (Table 4.4; Figure 4.1). Additionally, one set of four primers, without loop primers, flanking the RD4 was designed to detect *M. bovis* species (Table 4.4; Figure 4.1). Primer design was performed using the online Primer Explorer V4 software (<http://primerexplorer.jp/elamp4.0.0/index.html>) according to Notomi *et al.* [8]. Forward inner primers (FIP) were 5'-end labeled with either biotin or digoxigenin (Table 4.4). Oligonucleotide probes labelled with fluorescein isothiocyanate (FITC), complementary to the LAMP amplified DNA fragments, were also designed (Table 4.4). All primers and probes were synthesized as high performance liquid chromatography (HPLC)-grade by MWG Biotech (Germany).

Table 4.4. Sequences of LAMP primers and FITC-labelled probes.

Primer/Probe	Sequence (5'-3')	Complementary target
F3_ <i>IS6110</i>	TCA ACC GGG AGC CCA G	<i>IS6110</i> element of MTC
B3_ <i>IS6110</i>	TTT GCC GCG GGT GGT C	
FIP_ <i>IS6110</i>	Biotin-CGT AAA CAC CGT AGT TGG CGG CGC GCG ATG GCG AAC TCA	
BIP_ <i>IS6110</i>	GTG CCC GCA AAG TGT GGC TAA CAG TTT GGT CAT CAG CCG TTC	
LF_ <i>IS6110</i>	GAC GCG GCT GAT GTG CT	
LB_ <i>IS6110</i>	AGG TGG CCA GAT GCA CC	
FITC_ <i>IS6110</i>	TGA ACC GTG AGG GCA TC	

Table 4.4. (Cont.)

Primer/Probe	Sequence (5'-3')	Complementary target
F3_RD4flank	TTC CGA ATC CCT TGT GAA GT	RD4 flanking region
B3_RD4flank	CCC GTA GCG TTA CTG AGA A	
FIP_RD4flank	Dig-GGA AGG CGT CAT GAC CAA ACC ATT AAT GTG CGA GCT GAG CG	
BIP_RD4flank	GCC TACAAC GGC GCT CTC CAT TGA CCA GCT AAG ATA TCC GG	
FITC_RD4flank	ATT GTG AAT TCA TAC AAG CC	
F3_RD9	CCG TGC AAC GCA TCG G	RD9 (Rv2073c locus)
B3_RD9	GGC CAT CGT GCA CAC C	
FIP_RD9	Dig-TCC TCG GTC GCC GGG ATT CTC CAG GCC GGC TTT GG	
BIP_RD9	GAA CAC CAC CAG CGA TCC CCA CTA CGT CGC CCA GGT CAG	
LF_RD9	GCG CCA ACT ATG TCT ACG GAT C	
LB_RD9	CGC TGC CAG ATG AGT CAG C	
FITC_RD9	CGG CGG TGC GCA TCG	

Duplex LAMP reactions using labelled primers

LAMP reactions were carried out using labelled primers. Two separate LAMP reactions for the duplex detection of either MTC species and *M. tuberculosis*, and detection of either MTC and *M. bovis*, were optimized and carried out in a total volume of 25 µl containing 8 U/µl of *Bst* DNA polymerase (New England Biolabs), 1× Thermopol buffer (New England Biolabs), 1.4 mM of dNTPs (Promega), 6 mM of MgCl₂ (Promega), 0.8 M of betaine (Sigma-Aldrich), 0.2 µM of each external primers (F3 and B3), 1.6 µM of each internal primers (FIP and BIP), 0.8 µM of each loop primers (LF and LB, only for the amplification of IS6110 and RD9 targets), and 3 µl of extracted DNA template. LAMP amplification was assessed after the reactions with standard gel electrophoresis procedures.

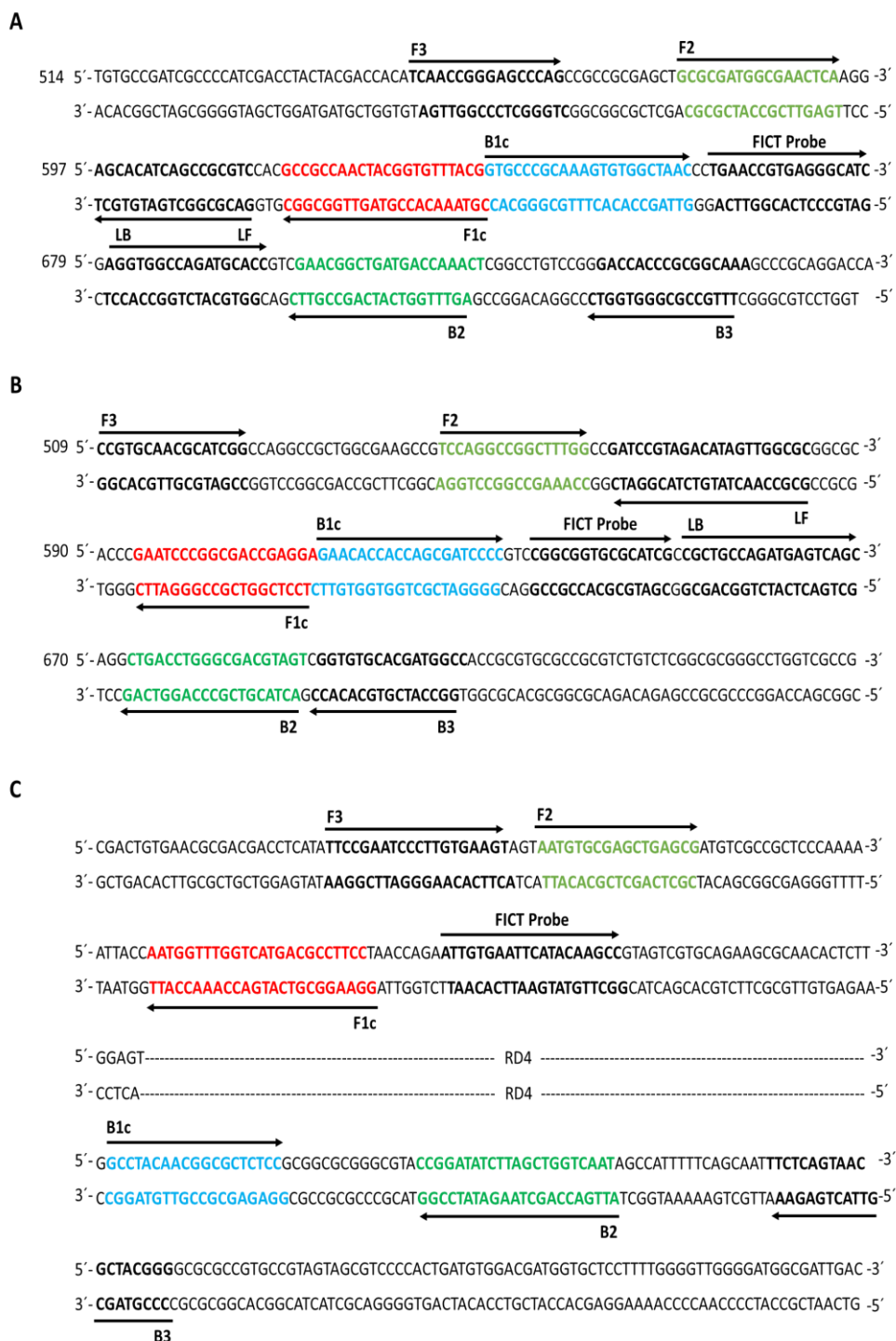


Figure 4.1. Location of the complementary regions of LAMP primers and FITC-labelled probes. Partial sequence of *M. tuberculosis* H37Rv IS6110 element (A), *M. bovis* AF2122/97 RD4 genomic region (B) and *M. tuberculosis* H37Rv RD9 genomic region (C), and location of the complementary regions used to design LAMP primers [F3, B3, FIP (F1c-F2), BIP (B1c-B2)], including loop primers for IS6110 and RD9 regions (LF, LB) and FITC-labelled probes. Arrows indicate the direction of extension.

Detection of LAMP products by LFD

For detection of LAMP products by LFD, 20 picomoles of the adequate FITC-labeled probe were added to the LAMP products. Probes were designed for the detection of IS6110 (MTC species), RD4 flanking region (*M. bovis*) and RD9 (*M. tuberculosis*) targets. After hybridization at 65 °C for 5 min, 8 µl of the hybridized product were added to 100 µl assay buffer in a new tube, and an LFD strip was dipped into the mixture for 2 min. Commercial universal LFD devices for the detection of labelled LAMP products were purchased from Milenia Biotec (HybriDetect 2T) and used according to the instructions of the manufacturer.

Analytical specificity and sensitivity of LAMP assays

To evaluate the analytical specificity of the two duplex LAMP-LFD assays, DNA samples from MTC and non-MTC strains were tested (Table 4.1). In order to assess the minimum DNA detection limit (analytical sensitivity), the two duplex LAMP-LFD assays were tested with ten-fold diluted template DNA, in concentrations ranging from 12.2 ng/µl to 12.2 pg/µl (for the DNA sample from *M. bovis* LNIV 7257), and from 12.5 ng/µl to 12.5 pg/µl (for the DNA sample from *M. tuberculosis* H37Ra).

Statistics

The kappa coefficient, with confidence intervals, for measuring the agreement between the results of the routinely performed identification of the isolates, and the dLAMP-LFD assays herein described, was computed using the clinical research calculators of the online VassarStats software (<http://vassarstats.net>).

4.4. Results

Design of LAMP primers and FITC probes

Based on the IS6110, RD4 flanking and Rv2073c (RD9) genomic regions of the MTC members, novel sets of LAMP primers and FITC probes complementary to the amplified regions were designed (Table 4.4; Figure 4.1). The complementary regions of

the LAMP primers set targeting the multi-copy element *IS6110* are shared by all MTC species (Figure 4.1A). The LAMP primers set targeting the RD4 flanking genomic region allow the amplification of products only if the RD4 deletion is present (as in *M. bovis*) (Figure 4.1B). The complementary regions of the LAMP primers set targeting the RD9 genomic region are located in the Rv2073c locus, coding a probable short chain dehydrogenase, which is present in *M. tuberculosis* but absent from *M. bovis* and from other MTC species (Figure 4.1C).

Duplex LAMP-LFD strategy

Two LAMP assays in duplex format combined with lateral flow dipsticks for assessing the amplification results were developed. One assay (dLAMP-LFD #A) targets MTC species and *M. tuberculosis* (with potential anticipated application mainly in laboratories dealing with the diagnosis of human TB). The assay allows the identification of the cultures as MTC members, by targeting their *IS6110* element, and as *M. tuberculosis*, by targeting their RD9 genomic region. The second assay (dLAMP-LFD #B) targets MTC species and *M. bovis* (with anticipated application in veterinary diagnosis laboratories). The assay allows the identification of MTC species through the amplification of *IS6110*, and the identification of *M. bovis*, by detecting the flanking region of RD4. The amplification of only the *IS6110* element, for both assays, means that we are in the presence of other MTC species beyond *M. tuberculosis* and *M. bovis*. Both assays were optimized for use with DNA templates extracted from mycobacterial cultures. DNA samples can also be extracted directly from BACTEC liquid culture bottles, using a simple boiling method.

Analytical specificity and sensitivity

LAMP assays were tested using DNA extracted from previously identified MTC and non-MTC reference and clinical strains as template (Table 4.1; Figure 4.2). For both dLAMP-LFD assays, the *IS6110* element was only detected in MTC species (Table 4.1; Figure 4.2). The dLAMP-LFD #B, targeting the RD4 flanking region, only detected *M. bovis* strains (Table 4.1; Figure 4.2A). The dLAMP-LFD #A, targeting the RD9 region, only detected *M. tuberculosis* strains (Table 4.1; Figure 4.2B). None of these genomic regions were detected in other strains not belonging to MTC.

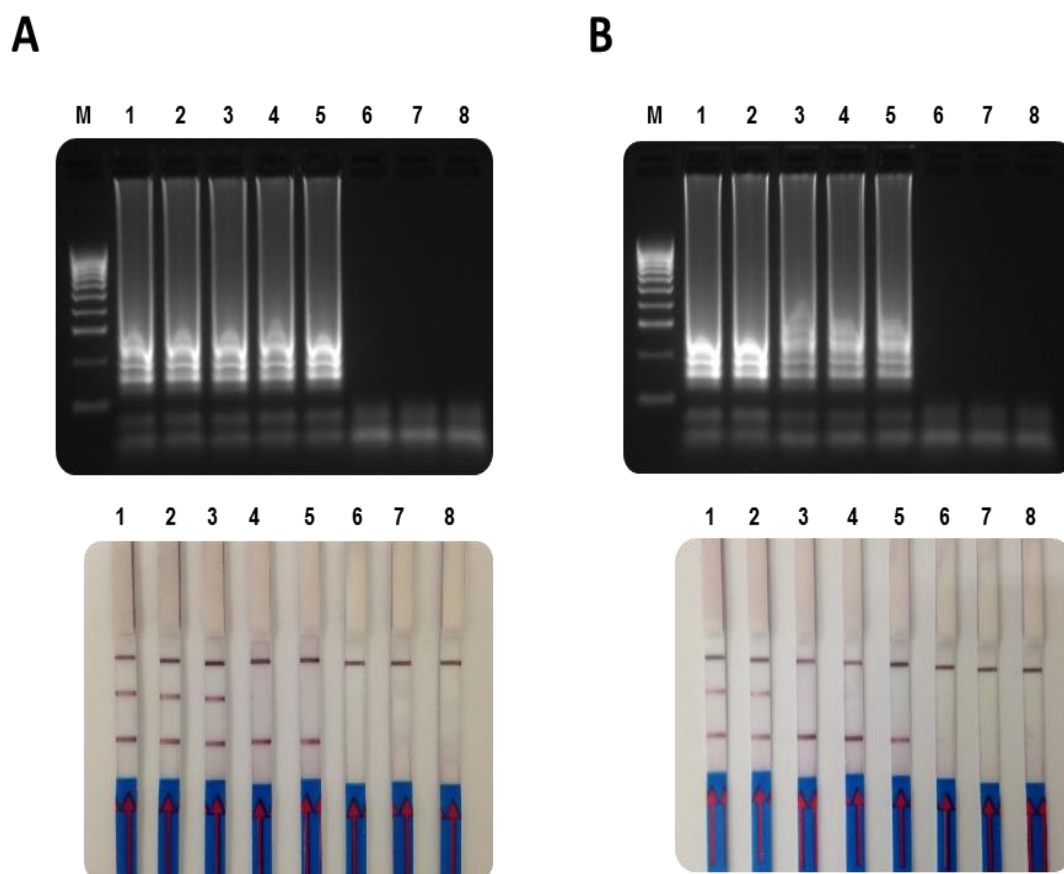


Figure 4. 2. Specificity tests of the dLAMP-LFD assays. (A) Amplification results of the dLAMP-LFD #B assay in an electrophoresis gel run (above) and in the lateral flow strips (below): 1 - *M. bovis* LNIV 7257; 2 - *M. bovis* LNIV 8016; 3 - *M. bovis* BCG ATCC 27291; 4 - *M. caprae* LNIV 17320; 5 - *M. tuberculosis* H37Ra; 6 - *M. avium* subsp. *avium* ATCC 25291; 7 - *M. avium* subsp. *paratuberculosis* LNIV 39888; 8 - H₂O. (B) Amplification results of the dLAMP-LFD #A assay: 1- *M. tuberculosis* LNIV 9605; 2 - *M. tuberculosis* H37Ra; 3 - *M. bovis* LNIV 7257; 4 - *M. caprae* LNIV 17320; 5 - *M. bovis* BCG ATCC 27291; 6 - *M. avium* subsp. *avium* ATCC 25291; 7 - *M. avium* subsp. *paratuberculosis* LNIV 39888; 8 - H₂O. Lane M represent DNA ladder marker. In the lateral flow strips, the first coloured test line (from the bottom) corresponds to the detection of the IS6110 element, the second test line to the detection of the specific fragment of either *M. bovis* (RD4 flanking region) or *M. tuberculosis* (RD9), and the third line is the flow control.

The results of the analytical sensitivity of the duplex LAMP assays were as follows: *M. tuberculosis* IS6110 and RD9 products were detected until the dilution containing 12.5 pg/μl of template DNA (Figure 4.3). A similar detection limit of 12.2 pg/μl was found for the assay targeting the IS6110 and the flanking RD4 region of *M. bovis* (Figure 4.3).

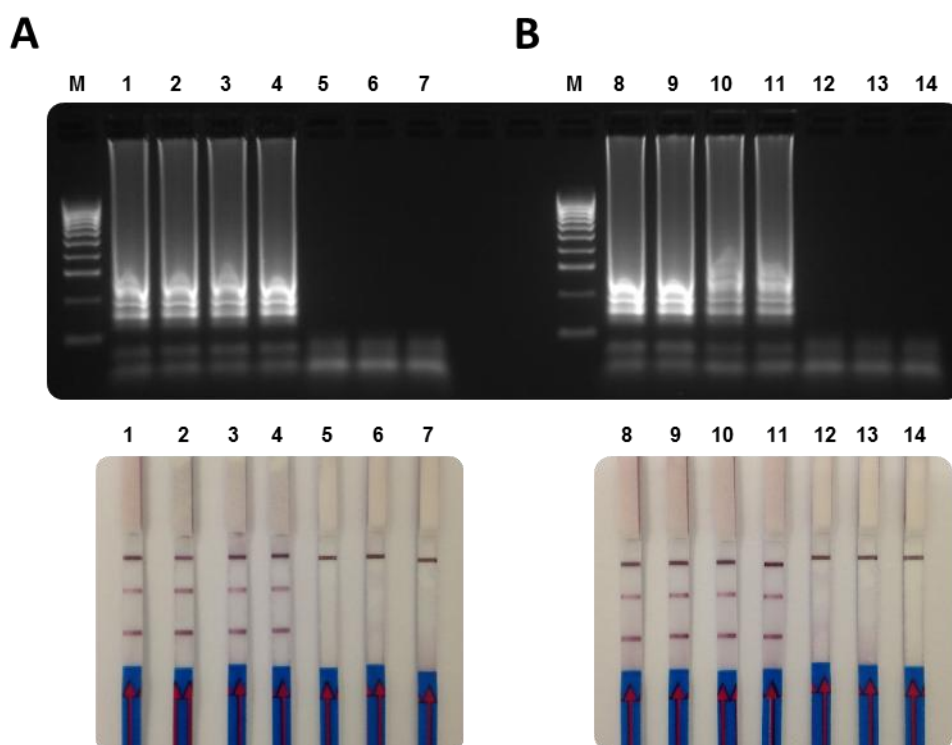


Figure 4.3. Detection limit of the dLAMP-LFD assays. (A) Results for the dLAMP-LFD #B assay in an electrophoresis gel run (above) and in the lateral flow strips (below): Ten-fold serial dilutions of *M. bovis* LNIV 7257 template DNA, ranging from 10^{-1} to 10^{-6} dilutions (lane 1 to 6) (initial concentration was 12.2 ng/ μ l). Lanes M and 7 represent DNA ladder marker and negative control (H_2O), respectively. (B) Results for the dLAMP-LFD #A assay in an electrophoresis gel run (above) and in the lateral flow strips (below): Ten-fold serial dilutions from *M. tuberculosis* H37Ra template DNA ranging from 10^{-1} to 10^{-6} dilutions [lane 8 to 13) (initial concentration was 12.5ng/ μ l). Lanes M and 14 represent DNA ladder marker and negative control (H_2O), respectively.

Testing of DNA templates extracted directly from BACTEC liquid cultures

The dLAMP-LFD #B, targeting the *IS6110* element and the flanking RD4 region, was tested using DNA extracted directly from a set of 117 sequential BACTEC liquid cultures, using a simple boiling method, obtained under the routine testing of TB-suspected tissue samples originally collected from bovines ($n = 69$), deer ($n = 15$) and wild boar ($n = 33$) (Table 4.3; Figure 4.4A).

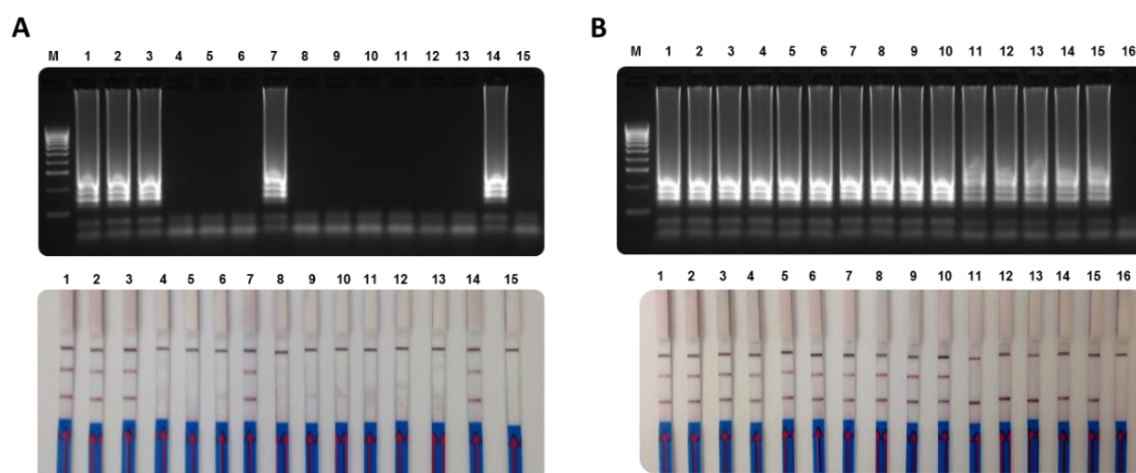


Figure 4.4. Examples of the dLAMP-LFD amplification results obtained with clinical isolates of animal and human origins. (A) DNA templates extracted from animal isolates tested with the dLAMP-LFD #B assay: 1 - *M. bovis* LNIV 7257; 2 - 37481/1; 3 - 37481/2; 4 - 35690; 5 - 37707/2; 6 - 31389; 7 - 37481/3; 8 - 41680/1; 9 - 31784; 10 - 31093; 11 - 31094; 12 - 31388; 13 - 31786; 14 - 33761; 15 - H₂O. (B) DNA templates extracted from human isolates tested with the dLAMP-LFD #A: 1 - IHMT 1; 2 - IHMT 2; 3 - IHMT 3; 4 - IHMT 4; 5 - IHMT 5; 6 - IHMT 6; 7 - IHMT 7; 8 - IHMT 8; 9 - IHMT 9; 10 - IHMT 10; 11 - IHMT 39; 12 - IHMT 46; 13 - IHMT 33; 14 - IHMT 34; 15 - *M. bovis* LNIV 7257; 16 - H₂O. Lane M represent DNA ladder marker. Amplification results in an electrophoresis gel run (above) and in the lateral flow strips (below).

Fifty five BACTEC cultures were identified as *M. bovis* by the routine bacteriological analysis and no bacterial growth was detected in 52 BACTEC samples. Ten samples were found to be infected with non-MTC isolates (Table 4.3). The IS6110 element and the flanking RD4 region were only amplified from all *M. bovis* infected cultures by the dLAMP-LFD #B assay, while all other samples yielded negative amplification results for both loci (Table 4.3). These results correspond to a perfect agreement with the results of the routine bacteriological analysis, with a kappa coefficient of 1 (CI_{P95%} 1.000).

Testing of DNA templates extracted directly from BACTEC liquid cultures

The dLAMP-LFD #A, targeting the IS6110 element and the RD9 locus, was tested using DNA extracted from a set of 50 mycobacterial human isolates, collected from sputum samples of individuals suffering with tuberculosis (Table 4.2; Figure 4.4B).

Forty cultures were identified as *M. tuberculosis* by the routine bacteriological analysis, six as *M. bovis* BCG and four as *M. africanum*. The duplex LAMP assay identified the forty *M. tuberculosis* samples as IS6110 and RD9 positive and the ten other MTC-belonging species as IS6110 positive and RD9 negative (Table 4.2). Therefore, the optimized assay also presented a perfect agreement with the results of the routine bacteriological analysis (Table 4.2), with a kappa coefficient of 1 ($CI_{95\%}$ 1.000).

4.5. Discussion

Pressing needs include more rapid diagnostic tests without sacrificing sensitivity, and point-of-care or point-of-need tests for use in low-resource settings and in-field situations. It is therefore critical to develop simple and affordable molecular tests for the above scenarios. In recent years, research has been focused on alternative methods to improve the diagnosis of pathogens. The invention of loop-mediated isothermal amplification (LAMP) has opened up a new horizon for molecular diagnosis. This novel alternative nucleic acid amplification method amplifies a target DNA under isothermal conditions, it is based on the principle of strand displacement DNA synthesis performed by a *Bst* DNA polymerase and production of stem-loop DNA structures under a constant temperature. The reaction relies on recognition of the DNA target by at least six independent sequences, making this assay highly specific. The method is rapid and has a DNA amplification efficiency equivalent to that of standard PCR-based methods. This method has drawn a lot of attention as it offers a fast, accurate, cost-effective, and user-friendly diagnosis for infectious microorganisms, do not require expensive equipments and all reactions can be performed in an isothermal environment [8]. To take LAMP to realistic point-of-need situations, lateral flow dipstick (LFD) technology could ease the detection of LAMP products in a closed environment. LFD works by immunochromatography. This method demonstrates high sensitivity and enables simple visual detection of the amplified DNA through an appearance of a red line in the test. A nucleic acid lateral flow assay is a prefabricated strip, consisting of a surface layer to carry the sample from the sample application pad via the conjugate release pad along the strip encountering the detection zone up to the absorbent pad, designed for diagnostic purposes to test the presence or absence of pathogens, in this case, DNA

targets that belongs to MTC species. In our nucleic acid lateral flow format, the LAMP products are labelled with one of two different tags (digoxigenin or biotin), which binds to tag-specific antibodies in distinct test lines of the strip assay.

Herein we implemented two Lateral Flow Dipstick assays, based on commercially available Milenia Biotec HybriDetect 2T® strips, combined with LAMP reactions able to detect and identify MTC-, *M. tuberculosis*- and *M. bovis*-specific genomic products. Two dLAMP assays were developed, the first dLAMP-LFD #A assay is able to identify MTC species and *M. tuberculosis* by the specific dual amplification of the IS6110 and RD9 genomic regions, respectively. The second dLAMP-LFD #B assay also detects the IS6110 region and, additionally, detects the absence of RD4 by amplifying the flanking regions (a *M. bovis*-specific feature). Both assays were tested with a wide range of MTC and non-MTC reference strains and clinical isolates, yielding an identification specificity of 100% for their target organisms. No cross-reactions to other mycobacteria were observed. The analytical sensitivities of the dLAMP-LFD assays were comparable to that of common standard PCR assays used for the amplification of MTB genomic targets.

By integrating the LAMP and LFD technologies, the analysis time of isothermal amplified products could be shortened in about an hour and a half, because of the elimination of the cumbersome electrophoretic step. In addition, the dLAMP-LFD assays can confirm the identity of the specific amplicons by hybridization with specific FITC-labeled probes and avoids the use of carcinogens, such as ethidium bromide reagent. In this study, the dLAMP-LFD assays were carried out in a total of 70 min (not including DNA preparation time), which was faster than typical PCR methods that require 2-3 h for the PCR cycling. The assays does not require expensive equipments, just a thermostatic water bath, enabling their use in low-resource settings and less equipped laboratories. The two optimized dLAMP-LFD assays represent a potential, rapid and reliable diagnostic tool useful or a point-of-need utilization, based in a user-friendly colorimetric reading of the DNA amplification results.

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CHAPTER 5

Final Conclusions and Perspectives

5.1. Final Conclusions

Diagnostic tests are key tools in the control of infectious diseases, which remain of the major challenges for human life. Molecular diagnosis, commonly known as Nucleic Acid Testing (NAT), is one of the most promising and exciting fields of the *In Vitro* Diagnostics (IVD) market, presenting a growth rate of about 20% per year. The market trend is the increasing utilization of NAT technologies, which seems also really promising for a more rapid and specific diagnosis of tuberculosis (TB), including animal TB. Molecular methods for detection and identification of species of the *Mycobacterium tuberculosis* complex (MTC) have undergone a great development in recent years, showing improvements in sensitivity, specificity and reproducibility. In addition to contributing to shorten the diagnosis of tuberculosis, these methods are also of great importance for the epidemiological study of this disease.

Most of the available animal and human TB diagnostic NAT assays are still ‘in house’ optimized for each laboratory, usually demanding highly qualified personnel and sophisticated equipments, albeit some expensive commercial applications exist (e.g. GenoType® MTBDR, Hain LifeScience). Furthermore, the development of NAT-based tools for studying animal TB and its agents is still in its infancy when compared to the already available assays for human TB. In this context, it is necessary that NAT technologies become simpler, standardized and affordable to make them effectively and widely used in routine clinical laboratories, including laboratories in the developing world where animal and human TB is still a huge scourge [1]. Consequently, this work focused on the study of new alternative and user-friendly NAT assays for the rapid and specific detection and identification of the most relevant MTC members in animal biological samples and in pure cultures.

The high genetic homology of MTC members reduces the number of unique genomic regions of each species, making it difficult to discriminate the members of the complex. In fact, the regions commonly used for identification of *Mycobacterium* spp., and bacteria in general, such as the sequences of the ribosomal DNA (rDNA) of 16 subunit (16S) and the intergenic 16S-23S rDNA [2], proved useless for the differentiation of species of the MTC. In addition to these, also the genes which nucleotide sequences allows the differentiation between species of MTC and non-MTC, such as the *hsp65* [3], *rpoB* [4, 5] and *dnaJ* [6] genes, have identical sequences among members of the MTC.

On the other hand, although the high genetic homology among MTC members, there is a considerable degree of polymorphisms associated with repetitive DNA, namely in insertion sequences (IS) and in the direct repeats region (DR), which appear useful for the identification and typing of MTC strains.

The analysis of the DR region by spoligotyping allows the differentiation between *M. bovis* and *M. tuberculosis*, besides the typing of the strains [7, 8]. However, the demanding execution and complexity of interpretation of patterns obtained by this technique prevent their use in routine use for discrimination of those species. Despite the difficulties caused by the high genetic homology between members of the MTC, the evolution of molecular methods and knowledge accumulated on the genomes of *M. tuberculosis* and *M. bovis* have contributed to the identification of several genomic regions used as target for the detection and identification of the species gathered in the complex. The *gyrB* gene encoding the B subunit of topoisomerase II is universally present among bacteria and the analysis of their polymorphisms is used in various bacterial genera for the discrimination of species and inferring of phylogenies [9]. The analysis of this gene polymorphisms by PCR-RFLP, using the restriction enzymes *RsaI*, *TaqI* and *SacII*, distinguishes the main MTC species, except *M. tuberculosis*, *M. africanum* subtype II and *M. canettii* [10, 11]. Goh and collaborators (2006) [12] introduced two enzymes, *HinfI* and *HaeIII*, to the algorithm proposed by Niemman *et al.* (2000) [11] for the differentiation of species of the MTC, which shows new restriction patterns, particularly for *M. canettii*, besides allowing the differentiation between *M. tuberculosis* and *M. africanum*.

Other regions, such as the *oxyR* pseudogene and *pncA* gene, the last one encodes the pyrazinamidase enzyme, present single nucleotide polymorphisms (SNPs) that allows the identification of *M. bovis*, without permit the differentiation from other members of the MTC [13, 14, 15]. In another example, the *hupB* gene, encoding a histone, only allows the differentiation between *M. tuberculosis* and *M. bovis* [16, 17]. The analysis of the *mtp40* gene, which encodes for a phospholipase C, has also been used to distinguish *M. tuberculosis* and *M. bovis*, being this gene absent in the second species [18]. However, the use of this gene is not appropriate for the discrimination between these species because several strains of *M. tuberculosis* have been identified in which the gene is also absent [19, 20].

Molecular tests used by laboratories to differentiate the species of MTC are usually implemented and standardized "in house". As far as we know, there is only one commercially available NAT test that can discriminate among the MTC members (Genotype MTBC, Hain Diagnostics, Germany). This is a reverse hybridization assay based on the polymorphism of sequences of the *gyrB* gene and the RD1 deletion of *M. bovis* BCG. However, in addition to being applicable only for the identification of mycobacteria in culture, this test is very expensive for a widespread use in routine diagnosis of tuberculosis.

It is noteworthy that real-time PCR technology has been extensively evaluated in the diagnosis of human tuberculosis, but there are scarce studies on its application to the diagnosis of animal tuberculosis [1, 21, 22, 23]. Therefore, in this work we described the development of two methods based on real-time PCR technology for the identification and differentiation of the most relevant MTC species (Chapters II and III).

Regarding bovine tuberculosis diagnostics, as mentioned in Chapter I, several molecular methods have been described over the years for the detection and identification of *M. bovis* in either clinical isolates or directly in histopathological sections or lesion samples (clinical samples). These methods are mainly based in sequences amplification by PCR. NAT assays based in the use of hybridization probes, despite having a sensitivity near 100%, usually require the presence of a high number of bacilli ($10^5/\text{ml}$) and are therefore mainly used for the identification of clinical isolates [24]. The PCR-based methods directly applied to histopathological and clinical samples have the advantage of allowing a much more rapid diagnosis, although the sensitivities of these methods rarely exceed 75% [17, 22, 23, 25-31]. The low sensitivity of molecular assays, when applied directly to clinical samples, is often caused by the presence of a small number of bacilli. In addition to the paucibacillary nature of the samples, also fibrosis and calcification of the lesions of bovine tuberculosis poses difficulties the DNA extraction of mycobacteria [25]. Amplification of DNA may be further compromised by the presence of a high ratio of eukaryotic genomic DNA [32]. Another major limitation of the PCR reaction is the presence of DNA polymerase inhibitors in the samples, giving rise to false negatives [26]. On the other hand, the occurrence of false positives due to cross contamination of samples during the processing is a commonly described problem in molecular techniques [33].

The amplification of insertion sequences specific of the species of MTC, including *IS6110* [34] and *IS1081* [35], do not allow the differentiation between the MTC species, but have the advantage to increase the sensitivity of detection of the complex due to the presence of multiple copies of these sequences in the genome. This is one major reason why, in Chapter II, we used one of these sequences as target for developing an alternative approach for enhancing MTC detection sensitivities directly from fresh animal tissues. An user-friendly DNA extraction protocol, mainly based in the use of simple commercially obtainable extraction kits, was coupled with an *IS6110*-targeted semi-nested real-time PCR assay that allows the direct detection of MTC members in animal tissue specimens with very high sensitivity, specificity and positive and negative predictive values, namely in bovine specimens [36]. With this approach we could solve the problem of the lack of sensitivity usually described by previous works.

Another recent molecular approach for the identification of MTC is based on the analysis of certain regions of difference in the genome of these species [37, 38]. As already mentioned in Chapter I, the pattern of presence or absence of certain RDs seems to reflect its evolutionary history, and has been useful to correctly identify the species of the complex [1, 21, 39, 40]. Also the distinction between the virulent and vaccine strains (BCG) is possible by the analysis of RDs, since RD1 is absent in BCG strain, in which genes involved in the production of virulence factors are located, namely the ESAT-6 protein [41]. In Chapter III we described a rapid and robust two-step five-target *TaqMan*[®]-based real-time PCR identification algorithm, based on genomic deletion analysis, to identify the MTC species most commonly associated with TB in livestock and other animals, namely *M. bovis* and *M. caprae*. The first step allows the confirmation of the cultures as MTC members, by targeting their *IS6110* element, or as a mycobacterial species, if only a 16S rDNA product is detected in the duplex amplification reaction. If a MTC member is identified, the second amplification step allows to assess the presence or absence of the RD1 (Rv3875 locus, *esat6*), RD4 (Rv1510) and RD9 (Rv2073c) genomic regions in the respective genome. The correspondent pattern allows to infer the species of the isolate as *M. tuberculosis* (if all RDs are present), *M. caprae* (if only RD1 and RD4 are present), *M. bovis* (if only RD1 is present) or as *M. bovis* BCG (if all regions are absent). The assay is significantly faster and simpler than other SNPs-targeted restriction analysis or reverse line probe

hybridization-based molecular assays, since it bypasses any post-amplification experimental steps and can be used with DNA templates extracted directly from liquid cultures using a simple, low-cost and fast boiling-based method. The assay is able to be further automated and implemented in the routine diagnostics framework of veterinary diagnostics laboratories, with a particular focus for reference laboratories.

The development of rapid and autonomous diagnostic methods, independent of the use of expensive or sophisticated equipment, is also very relevant. The PCR reaction is a limiting factor in this regard. Consequently, as already mentioned above in this dissertation, it is quite interesting to study the possibilities of replacing this step in the experimental procedures for the identification of members of the *Mycobacterium tuberculosis* complex. Particularly interesting to analyse are the techniques of isothermal amplification and detection of specific nucleic acids. The application of these simplified methods will be particularly interesting for use in laboratories with fewer resources and in developing countries, where tuberculosis is still one of the most important cause of morbidity and mortality. Isothermal DNA amplification processes could facilitate their integration into bench NAT kits independent of the utilization of sophisticated equipment. One such technology is Loop-Mediated Isothermal DNA Amplification (LAMP) [42, 43, 44]. LAMP relies upon an auto-cycling strand displacement DNA synthesis, being also more tolerant to the presence of inhibitory substances [43]. The high potential of LAMP for the development of improved NAT kits fully justifies the increasing number of reports on its utilization, including for the detection of *M. tuberculosis* in sputum [45, 46, 47]. In Chapter IV, a multiplex LAMP-based assay was proposed, coupled to lateral flow dipstick technology for the detection and differentiation of MTC members. Two Lateral Flow Dipstick assays were implemented, based on commercially available Milenia Biotec HybriDetect 2T[®] strips. These strips were combined with LAMP technology and the assays were able to identify MTC-, *M. tuberculosis*- and *M. bovis*-specific genomic products obtained in duplex isothermal DNA amplification reactions. The multiplex utilization of these primers allowed not only the detection of MTC species, but also the discrimination between the main two members of this complex, *M. bovis* and *M. tuberculosis*. The first assay is able to identify MTC species and *M. tuberculosis* by the specific dual amplification of the IS6110 and RD9 genomic regions, respectively. The second assay also detects the

IS6110 region and, additionally, detects the absence of the RD4 region (a *M. bovis*-specific feature). Our system involved two steps. The first step involves the utilization of the designed primer sets in a multiplex format, leading to the LAMP-based amplification of the respective DNA fragments from the MTC members. Templates for the reaction can be extracted DNA from purified MTC cultures from bovine samples. In the second step, the amplified products are colorimetrically identified by lateral flow dipstick technology. This NAT strategy may be the basis of an autonomous, user-friendly and affordable diagnostic kit for the detection and identification of tuberculous mycobacteria.

During the course of this work, we had the opportunity to investigate new evidences on the circulation of MTC species in wild carnivores from mainland Portugal, in a parallel study where we could apply the nested real-time PCR assay described in Chapter II. Animal tuberculosis, caused by MTC bacteria, continues to affect domestic and wild mammals in Iberia, with the potential to influence the structure and stability of natural communities. In Portugal, the indicators of the bovine TB eradication plan demonstrate an incidence increase, with new outbreaks and areas of high prevalence, which have been partially associated with the sylvatic cycle of *Mycobacterium bovis* and the potential overlap of wildlife habitat with cattle under extensive husbandry. The increase of the abundance, spatial aggregation and artificial management of wild ungulates in touristic areas of big game hunting, as well as facilitating hunting practices, may increase the risk of transmission to other species and undermine the efforts associated with eradication campaigns. Therefore, we had the opportunity to integrate a larger consortium studying these topics, where we used our nested real-time PCR assay to assess the presence of MTC-associated DNA in 268 tissue samples collected from a set of six species of widely distributed wild carnivores from mainland Portugal. The tissues (lung, liver, and lymph nodes) were obtained during necropsy of road-killed animals or animals collected in corrective actions of predators. Despite the high degree of autolysis of a significant part of the tissues and the absence of gross lesions, suspect microscopic lesions were identified in the liver and lungs of foxes and Egyptian mongooses that can be grouped into two types: one, characterized by microgranulomas with mononuclear cells, and another with lesions of varying intensity and specificity, characterized by small foci of infiltrating mononuclear cells. *M. bovis* was isolated from the tissues of

two Egyptian mongooses. The presence of MTC-specific DNA was assessed using DNA extracted from the tissues as template for the semi-nested real-time PCR. Our assay revealed that around 10% of the tissue samples were infected with MTC species, with a particular emphasis for samples from foxes and mongooses. These preliminary results suggest that MTC circulates in wild carnivores of mainland Portugal and that the host spectrum affected by those agents is increasing. These results will soon be integrated and published in a broader study, aiming the clarification of the presence of tuberculosis in sylvatic animals in Portugal.

At the end of this work we have demonstrated the development of novel *post-mortem* NAT assays for the laboratorial diagnosis of bovine TB and for the discrimination of MTC members. Two methods based on real-time PCR technology, a semi-nested real-time PCR assay for the direct detection of MTC in animal samples and an identification algorithm based on TaqMan[®] technology to identify the key members of MTC, and a third method based on LAMP coupled to Lateral Flow Dipstick (LFD) technology, have been developed. The first method allowed the identification of species belonging to MTC, the other two methods allowed the identification and differentiation of the most relevant members of the complex. These methods have been tested with reference strains and clinical isolates, proving to be highly sensitive and specific. The work herein described aimed to make a small contribution to the improvement of the molecular diagnosis of animal tuberculosis and to an accurate identification of the most relevant species of the MTC. The results presented are the basis for the setting of improved diagnostic NAT kits that will surely represent a potential breakthrough with expected high impact on the improved diagnosis and control strategies of animal tuberculosis.

5.2. Perspectives

More than to repeat and discuss the results obtained in this work, we intend, in these concluding remarks, to raise some proposals and modestly contribute to the launch of some clues for future work. As already mentioned, the species belonging to MTC are genetically very similar but differ in their epidemiology and antimicrobial susceptibility characteristics. *M. bovis* and *M. caprae* also represents a high potential for zoonotic transmission to humans [48, 49], with evidence for the possibility of person-to-person

transmission [50]. However, the main routes of transmission are the contact with infected animals and ingestion of unpasteurized dairy products. These zoonotic MTC species are responsible for up to 7.2% and 15% of the human TB cases in industrialized and developing countries, respectively [51]. Studies on the prevalence of human TB caused by these species in Portugal are scarce and it is still unknown the zoonotic potential of animal-associated members of the MTC as agents of human TB in Portugal. It would be interesting to perform a retrospective analysis of a large collection of MTC isolates from patients with TB, which will allow to account for representative strains of *M. bovis* and *M. caprae* responsible for any cases of human TB and to assess the public health impact of these zoonotic mycobacteria in Portugal. The NAT tools developed in this work will be useful to track any past or current outbreaks of human TB of zoonotic origin in Portugal. The strains of *M. bovis* and *M. caprae* could be genotyped by spoligotyping and MIRU-VNTR and profiles of the human-associated isolates could be compared with the ones of animal origin circulating in our country, in order to evaluate their potential zoonotic origin. Achieving this is only possible with the intimate cooperation of research units devoted to animal and human health.

Another interesting topic is the development of alternative approaches for the extraction of mycobacterial DNA from bovine tissue samples. Immunomagnetic capture techniques to selectively separate and physically concentrate MTC cells from infected tissues could be an appropriate proposal. Previous preliminary works exploring this approach to recover *M. bovis* or *M. tuberculosis* cells from soil [52], cerebrospinal fluid [53] and bovine tissues [54, 55, 56] showed some promise but improvements are needed to allow its wider use in routine diagnostics. The improvement of the DNA extraction procedures from tissues will allow an easier application of our multiplex real-time PCR (Chapter III) and dLAMP-LFD (Chapter IV) assays to the direct analysis of animal tissues samples. This will be important to improve the diagnosis of animal tuberculosis and consequently contribute to a more effective control and eradication of the disease in our country.

In a previous work, we developed an alternative concept for the colorimetric identification of relevant MTC members based in the detection of their PCR-amplified *gyrB* gene using species-specific gold-nanoprobes [57]. It was not possible, in the current work, to continue exploring this concept. However, it would be very interesting

to continue improving this technology regarding three main aspects. First, on the design of novel sets of gold-nanoprobes that will allow the identification of more MTC species, namely of *M. caprae*, and reinforce the identification of *M. tuberculosis* and *M. bovis*. Besides the *gyrB* gene, the already referred RDs and DRs could be used as targets [37], allowing a more efficient and robust species discrimination. Secondly, investigate the possibility to replace the PCR step of the assay by an isothermal DNA amplification technology, such as LAMP [58]. Thirdly, optimize the assay for the analysis of mycobacterial DNA extracted directly from presumptive infected tissue samples.

Finally, it's our purpose to contribute to the enormous task of controlling and preventing the spread of human and animal TB.

5.3. References

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